MOLECULAR INSECT SCIENCE

Edited by
H. H. Hagedorn,
J. G. Hildebrand,
M. G. Kidwell,
and
J. H. Law

MOLECULAR INSECT SCIENCE

MOLECULAR INSECT SCIENCE

Edited by
H. H. Hagedorn,
J. G. Hildebrand,
M. G. Kidwell,
and
J. H. Law
Center for Insect Science
University of Arizona
Tucson, Arizona

Springer Science+Business Media, LLC

Library of Congress Cataloging-in-Publication Data

International Symposium on Molecular Insect Science (1989 : Tucson, Ariz.)
Molecular insect science : [proceedings of the International Symposium on Molecular Insect Science, held October 22-27, 1989, in Tucson, Arizona] / edited by H.H. Hagedorn ... [et al.].
p. cm.
Includes bibliographical references and index.

1. Insects--Physiology--Congresses. 2. Insects--Genetics--Congresses. 3. Molecular biology--Congresses. 4. Molecular genetics--Congresses. I. Hagedorn, H. H. II. Title.
QL495.I554 1989
595.7'08--dc20 90-46474
CIP

Proceedings of the International Symposium on Molecular Insect Science,
held October 22–27, 1989, in Tucson, Arizona

ISBN 978-1-4899-3670-7 ISBN 978-1-4899-3668-4 (eBook)
DOI 10.1007/978-1-4899-3668-4

Plenum Press, New York

PREFACE

This volume contains the scientific papers and abstracts of posters presented at the International Symposium on Molecular Insect Science held in Tucson, Arizona, October 22-27, 1989. This meeting was organized by the Center for Insect Science at the University of Arizona in response to the growing need for a forum dedicated to the impact of modern biology on insect science. While scientific studies of a few insects, notably *Drosophila melanogaster*, have always had a central role in the development of biology, it is only recently that tools have become available to extend these studies to other insects, including those having economic and medical importance. The Tucson meeting was evidence of how far we have come in extending modern biological tools to the study of insects. It is also evident from the contents of this book that the study of insects is making an increasingly important contribution to the advancement of biology generally. Given the large impact of insects on human life, such a development has considerable importance for human welfare, and of the welfare of the ecosystem as a whole.

It should be noted that several of the participants who presented posters were invited to prepare full length papers to ensure that the book covered the major areas of insect science.

The financial support of the National Science Foundation and the Monsanto Corporation is gratefully acknowledged. Thanks are also due to Sharon Richards for her dedicated work on the manuscripts.

Henry H. Hagedorn
October 1990

CONTENTS

I. Genome Characterization

II. Gene Isolation, Characterization and Regulation

III. Insect-Virus Interactions

IV. Proteins of the Hemolymph

V. Peptide Hormones

VI. Pheromones

VII. Cytochrome P450

MOLECULAR CHARACTERIZATION AND GENOME MANIPULATION OF THE MOSQUITO, *Aedes aegypti*

Julian M Crampton, Alison Morris, Gareth Lycett,
Ann Warren and Paul Eggleston

Wolfson Unit of Molecular Genetics, Liverpool School of Tropical Medicine
Liverpool, U.K. L3 5QA

INTRODUCTION

Diseases transmitted by insects, and particularly by mosquitoes, remain some of the most important health problems in the world today. Malaria, transmitted by Anopheline mosquitoes affects approximately 489 million children per year (Stürcher, 1989). *Aedes aegypti* is the major urban vector of the arboviruses including yellow fever, dengue and dengue haemorrhagic fever (DHF). These acute diseases also affect millions of people and cause many fatalities. Urban epidemics occur frequently, making DHF a major cause of hospitalization and death of children in many Asian countries (Rudnick, 1967).

A number of chemical, biological, environmental and genetic techniques are currently employed to control insect vector populations, but, to a large extent, control has relied on the elimination of breeding sites and the application of chemical agents. There are increasing problems associated with the use of synthetic insecticides for vector control, including the evolution of resistance, the high cost of developing and registering new insecticides and awareness of pollution from insecticide residues. These factors have focussed attention on the need for alternative measures, such as biological control using *Bacillus thuringiensis*. Clearly, the usefulness of genetic manipulation as an approach to insect control has first to be assessed, but it may have potential both for population suppression and for altering the vectorial capacity of insect populations.

Initially, there is a need for a much greater understanding of the basic molecular biology of mosquito disease carriers. If mosquitoes are to be manipulated in a controlled and directed fashion they must become as well known as *Drosophila melanogaster*. A means of introducing manipulated DNA into mosquitoes is also needed, as well as an efficient DNA vector system which will allow stable integration of the introduced DNA. The DNA which is introduced may not only have to be expressed, but should also provide a means of selecting the transformed individuals. Subsequently, potential target genes have to be identified that are suitable for manipulation or disruption. Finally, it would be naive to assume that the problems would be solved by the introduction of foreign genes into laboratory mosquitoes. There would be considerable practical and perhaps ethical problems associated with the release of genetically manipulated mosquitoes into natural populations. Despite these difficulties, genetic transformation has much to offer, both to basic mosquito research and to the development of future control schemes. Compared with classical mosquito genetics, two particular advantages are evident. One is the potential to exploit genes across species barriers and the other the ability to introduce particular, defined DNA sequences without the genomic disruption of a conventional cross.

We and others have concentrated on the mosquito, *A. aegypti*. The importance of this insect as a disease carrying vector coupled with its suitability for laboratory research and its potential for studies in genetics (Knipling et al., 1968) has made it the focus of research in mosquito molecular biology.

Molecular Insect Science, Edited by
H. H. Hagedorn *et al.*, Plenum Press, New York, 1990

Here, we describe some aspects of the progress which has been made towards characterising the genome of *A. aegypti* and developing the systems necessary for the successful manipulation of this mosquito genome by micro-injection of embryos and transfection of cell cultures.

MOLECULAR CHARACTERIZATION OF THE *Aedes aegypti* GENOME

Until recently, very little was known about the size and organisation of mosquito genomes. Using DNA reassociation kinetics, the total genome size and complexity and the individual complexities of the various components of genomic DNA have been determined for both *A. aegypti* cell line and larval DNA.

Renaturation of genomic DNA was monitored using S1 nuclease analysis coupled with TCA precipitation (Britten et al., 1976). DNA was sheared by sonication to a mean size of 450bp. DNA fragments were ethanol precipitated and resuspended in 0.12 M phosphate buffer pH 7.0 at concentrations of 10 μg DNA/ml for low C_0t values, 100 μg DNA/ml for mid C_0t values and 1mg DNA/ml for high C_0t values. Tracer DNA was prepared by nick translation of genomic DNA to a specific activity of 10^8 dpm/μg DNA. Driver and tracer DNAs were mixed and denatured by boiling for 10 mins. Renaturation was performed at 63^0C. Forty samples were taken at timed intervals to cover Log C_0t values ranging from -2.7 to 3.9 (Fig. 1). To estimate the total genome and individual component complexities it was necessary to determine the $C_0t_{1/2}$ value of a single copy sequence of known complexity using the above conditions of denaturation and analysis. For this reason, *E. coli* DNA was used as a standard.

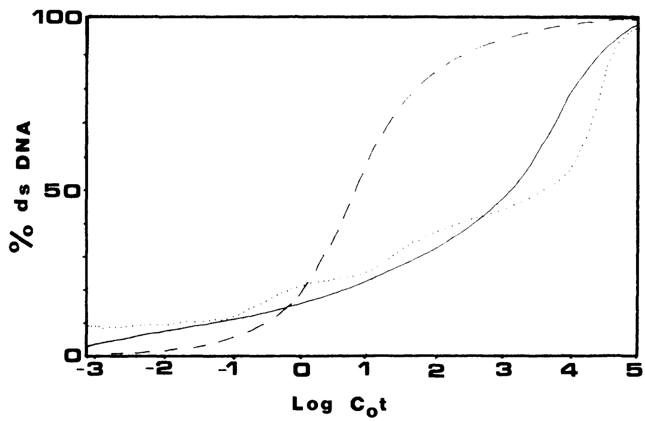


Fig. 1. Complexity analysis of *A. aegypti* genomic DNA. C_0t curves following renaturation of sheared *A. aegypti* larval DNA (——), MOS20 cell line DNA (.......) and *E. coli* DNA (– –). The line of best fit for C_0t values was determined using a non-linear regression program adapted for DNA reassociation kinetics followed by S1 nuclease analysis.

Data were analysed using a non-linear regression program adapted for DNA reassociation kinetics followed by S1 nuclease analysis. This allows analysis of the separate components of genomic DNA eg. single copy, middle repetitive (MR) and highly repetitive DNAs (Green et al., 1982). Comparison of the Cot data for *A. aegypti* cell line and larval genomic DNAs reveals a 2-fold difference in the estimated genome size, being 1.5 x 10^9bp and 8.0 x 10^8bp respectively (Table 1). This variation may reflect changes in the chromosomal content of cultured cells involving such phenomena as aneuploidy (Freshney, 1986). It may also be a reflection of elevated levels of replicative transposition of endogenous transposable genetic elements (TGEs) such as observed in *D. melanogaster* following the cultivation and passage of embryonic cells (Potter et al., 1979).

Studies on genome size and organisation in the mosquito *A. albopictus* (Black and Rai, 1988) have led to the suggestion that intraspecific variation in genome size is primarily due to differences in the amounts of highly repetitive (HR) DNA. However, preliminary find-

ings (Table 1) indicate that the MR fraction of the genome plays an equally important role in intraspecific variation in genome size of the mosquito. Technical and analytical errors inherent in monitoring the DNA reiteration of complex genomes will affect the estimated values of genome components. However, our data suggest that the values for size and complexity of the total genome and individual DNA components presented here can, at least, be taken as good estimates. Furthermore, they have indicated the parameters necessary for the purification of the separate components of the genome.

Table 1. Analysis of the different components present in *Aedes aegypti* Bangkok larval and MOS20 cell line DNA.

	BANGKOK LARVAL DNA	MOS20 CELL LINE DNA
Total Genome size (bp)	8.0×10^8	1.5×10^9
Picograms DNA/cell	0.83	1.5
Proportion of Genome		
HR DNA	0.2	0.13
MR DNA	0.2	0.24
SC DNA	0.6	0.64
Total base pairs		
HR DNA	1.6×10^8	1.4×10^8
MR DNA	1.6×10^8	2.6×10^8
SC DNA	4.8×10^8	1.1×10^9
Average reiteration frequency		
HR DNA	$10^5 - 10^6$	10^5
MR DNA	10^3	10^3
SC DNA	1	1

This study, together with filter hybridisation experiments using an *A. aegypti* genomic library probed with total genomic and rDNA probes, has indicated that the genome of *A. aegypti* is both large and complex and exhibits a short period interspersed pattern (SPI) of repeat sequences. Thus, for example, short sections of repetitive DNA are interspersed regularly with regions of unique sequence, or coding DNA. Patterns of sequence arrangement and genome complexities have important implications in experimental design when attempting to isolate DNA sequences. For example, highly repetitive sequences within genomic clones containing large DNA inserts from an organism with an SPI arrangement of repeats may well mask the hybridisation characteristics of sequences of interest, eg. low MR or unique sequences, particularly when probing with complex genomic DNA probes.

MICRO INJECTION OF *Aedes aegypti* EMBRYOS

Germ line transformation requires the introduction of a eukaryotic DNA transformation vector, capable of precise integration at a relatively high frequency, into the developing insect embryo. The use of the *D. melanogaster* transposable genetic *P* element in the successful transformation of other species of *Drosophila* suggested originally that there may be no species barrier to the use of this element (Brennan et al., 1984; Daniels et al., 1985). Although there is now evidence that the *P* element may not behave as a high efficiency transposon in evolutionarily distant organisms (O'Brochta and Handler, 1988) we decided to use the well characterised *P* element vector-helper system to establish transformation in *A. aegypti*.

Unlike that of *D. melanogaster*, the rigid, opaque endochorion of the mosquito embryo cannot be removed, and the embryos are extremely sensitive to desiccation (Fig. 2a). To overcome these problems, an injection needle must be used which is capable of penetrating the rigid endochorion without tearing it (Fig. 2b). Glass capillaries with tips of 100-300 µm x 3-10 µm have been used successfully. Exposure of 30s. to 1 min. to the atmosphere appears to provide adequate desiccation, leaving the embryos viable and yet able to incorporate the injected DNA solution without damage. Following this, further desic-cation is prevented by covering the embryos with water-saturated halocarbon oil which permits the normal uptake of water by the developing embryos until they are returned to standard insectary conditions (Morris et al., 1989).

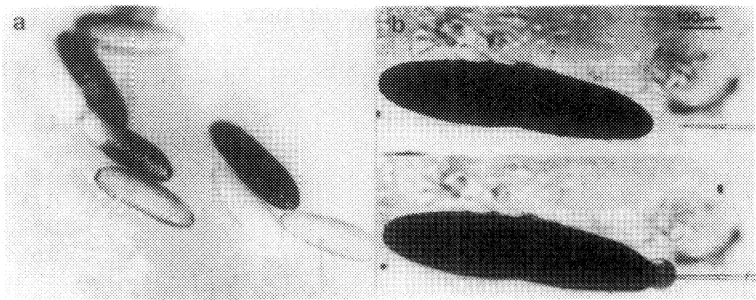


Fig. 2. (a) Early *Aedes aegypti* embryos (<4 hrs) at various stages of development. The change from white to black is complete after 4 hours. (b) Microinjection of an *A. aegypti* embryo

Having successfully introduced *P* element DNA into the embryos, with survival rates comparable to those obtained in work with *D. melanogaster* (Spradling and Rubin, 1982), it is then necessary to study the fate of this DNA. The *P* element introduced into the mosquito genome is engineered to carry an antibiotic resistance gene which acts as a dominant selectable marker for the identification of transformed individuals, and a multiple cloning site to facilitate the introduction of specific DNA sequences. The *P* element inverted terminal repeats are joined by a 500bp sequence from the *white* eye locus of *D. melanogaster* (Fig. 3a). It has been shown that this may be used as a probe to distinguish between random integration events and those resulting from the precise excision and transposition of the *P* element (Miller et al., 1987). A precise *P*-mediated event would involve only those sequences within, and including, the inverted repeats and would exclude the *white* locus DNA. A helper plasmid is co-injected with this vector plasmid, in order to supply a functional *P* element transposase. The helper, however, has a deleted inverted terminal repeat which is known to prevent it from integrating into the recipient genome (Steller and Pirrotta, 1985) (see Fig. 3b).

Labelled vector plasmid is used to probe genomic DNA digests of putative transformants and their progeny (Morris et al., 1989; Miller et al., 1987). Intact vector plasmid has been detected in 5-10% of the adults arising from injected embryos (G_o) confirming that the DNA is not immediately broken down by the mosquito. Genomic integration of vector DNA has been detected in one G_o individual, probably in somatic tissue (Morris et al., 1989). Although the aim is to modify the germline, this does provide encouraging evidence that the *P* element vector-helper transformation system may be operational within the genome of *A. aegypti*. Additional integration events have been isolated subsequently from the G_1, G_2 and G_3 generations following transformation (Fig. 4). These events are heritable and clearly involve the germ line of the mosquito, although several of them have proved to be unstable from one generation to the next. Such integrations are apparently independent of the *white* locus DNA of *D. melanogaster* and may represent *P*-mediated transposition events (Morris et al., 1989). Further integration events are currently being analysed in order to determine their precise nature. Similar approaches have been described for *Anopheles gambiae* (Miller et al., 1987) and *A. tri-seriatus* (McGrane et al., 1988).

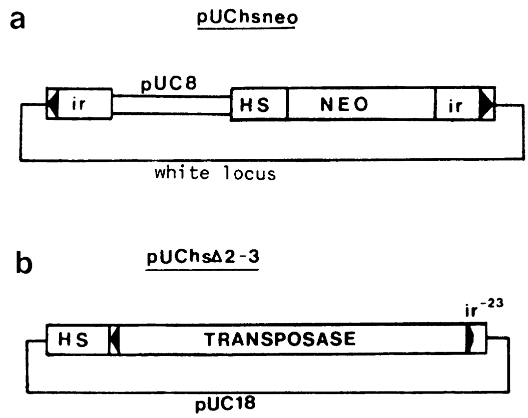


Fig. 3. (a) The pUChsneo transformation vector carries an antibiotic resistance gene (*neo*) controlled by a *Drosophila* heat shock promoter hsp70 (HS) and a multiple cloning site within the inverted terminal repeats of the *P* transposable element. The *P* sequences are linked by 500bp of *white* locus DNA from *Drosophila*. (b) The pUChs π(δΔ2-3) helper plasmid carries the entire *P* transposase coding sequence which is transcribed under the control of the *Drosophila* hsp70 heat shock promoter (HS). The sequence has been modified by *in vitro* removal of an intron to overcome the germ line specificity observed in *Drosophila*. A deletion of one of the inverted terminal repeats prevents the transposition mediated integration of helper sequences following micro-injection.

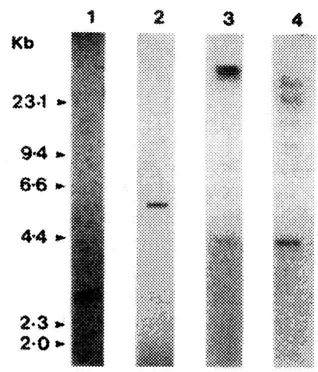


Fig. 4. Instability of transformed sequences in subsequent generations. Southern transfer of HindIII digested genomic DNA from transformed individuals probed with the labelled pUChsneo plasmid. Lane (1) is a G_1 transformed adult. Lanes (2, 3 & 4) are G_2 individuals arising from the G_1 parent shown in (1). Following hybridisation, the filters were finally washed in 2 x SSC, 0.1% SDS at 42^0C (1, 2) or 72^0C (3, 4). Autoradiography was at -70^0C for 4 weeks (1) or 2 weeks (2, 3 & 4).

Analysis of the expression of *P* element transposase within the mosquito, together with assays for the precise excision and integration of mobile DNA will ultimately determine the usefulness of the *P* element as a transformation vector in insects other than *D. melanogaster*. Indeed, even if other (non-*P* element encoded) proteins are found to be essential for transposition in *D. melanogaster*, we envisage that the appropriate genes will eventually be isolated thereby allowing them to be co-injected with the *P* element vector into the mosquito embryo.

TRANSFORMATION OF *Aedes aegypti* CELLS IN CULTURE

The transfection of cells in culture can give many insights into the molecular biology of an organism. The simple, controlled environment of cultured cells allows one to follow the expression of cloned genes, and so delineate promoter and enhancer sequences. Genetically manipulated cell cultures are also able to over-produce specific proteins which facilitates their isolation and purification.

We have used cultured mosquito cells to examine different transfection vectors and to establish a suitable system for germline transformation of *A. aegypti.* Initial experiments have utilised *P* element vectors developed for the genetic manipulation of *D. melanogaster.* DNA has been introduced into several cell lines by a variety of techniques devised to generate transient cell membrane pores, including calcium phosphate precipitation (Wigler et al., 1977), dextran sulphate (Lopata et al., 1984), polybrene (Durbin and Fallon, 1985), electroporation (Chu et al, 1987) and lipofection (Felgner et al., 1987). Polybrene and electroporation mediated transfection have proved to be most successful in *A. aegypti* cultures. A number of cell lines have been successfully transformed to antibiotic resistance using these techniques and the nature of the transformation is currently being defined.

THE SEARCH FOR TRANSPOSABLE ELEMENTS IN *Aedes aegypti*

It may be that a universal transformation system functioning in all organisms is not possible and that endogenous transposable elements will need to be isolated for each particular species and manipulated to produce suitable transformation vectors. Two approaches were initially envisaged for identifying TGEs in the *A. aegypti* genome. The first was to analyse specific gene systems, in particular the rDNA and the *white* eye locus, in an attempt to isolate variants of these genes which may have arisen from the insertion of TGE's. Most of this work has concentrated on the rDNA which has a repeat unit of 9.0 kb in *A. aegypti.* Individual rDNA repeats exhibit a high degree of homogeneity with respect to length and the position of restriction enzyme recognition sites. Preliminary mapping data together with partial digestion experiments demonstrate that, as in all other higher eukaryotes, the rDNA repeats are arranged in a head-to-tail, tandemly repeating manner (Gale and Crampton, 1989).

The restriction mapping of cloned rDNA repeats has confirmed that they are largely of uniform length in *A. aegypti* and has provided a more detailed physical map of the DNA. Transcription mapping has allowed the allocation of identities to the various restriction fragments and the approximate positioning of the transcription unit (Fig 5). The estimate of rDNA repeat copy number in *A. aegypti* (approximately 500 copies per haploid genome) is similar to that of 430 copies per haploid genome reported for the closely related species, *Aedes albopictus.* Ribosomal DNA thus comprises approximately 0.6% of the total *A. aegypti* genome.

An analysis of the variation between strains of *A. aegypti* has served to confirm the remarkable homogeneity of the rDNA repeat unit in this insect (Fig. 6a). A small number of variants have been isolated from an *A. aegypti* genomic library and mapping shows that what variation there is derives from restriction enzyme site polymorphisms (Fig. 6b). It would appear therefore that there are very few, if any, insertions of mobile elements in the rDNA of *A. aegypti.*

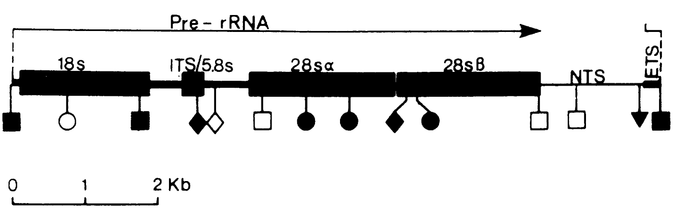


Fig. 5. Restriction map and transcribed regions of the *A. aegypti* cloned rDNA repeat. (■) *Eco*RI; (♦) *Pst*I; (□) *Kpn*I; (▼) *Hin*dIII; (○) *Pvu*II; (◊) *Sac*I; (●) *Hin*cII.

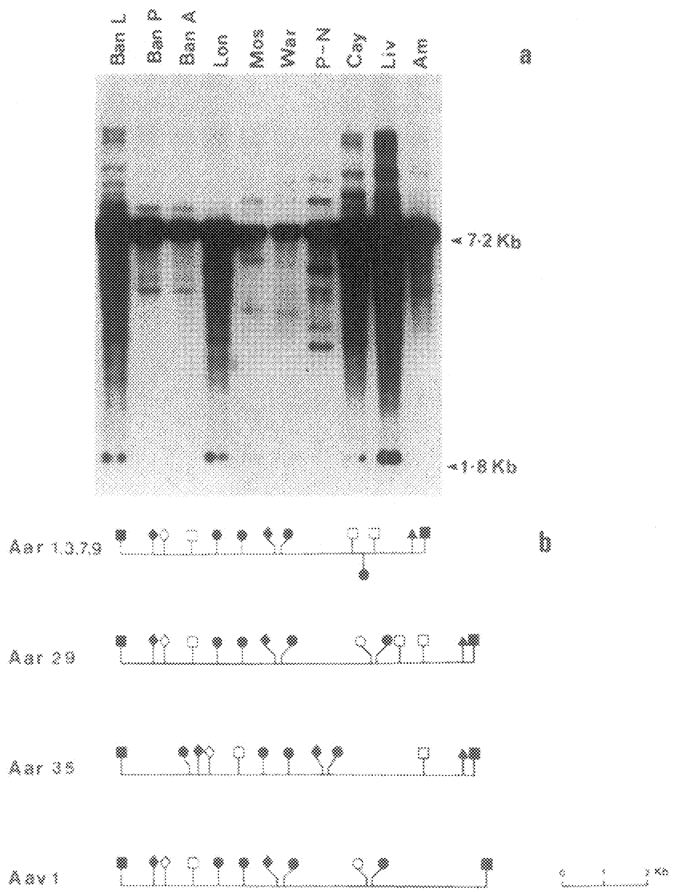


Fig. 6. Variation in the rDNA repeat in *Aedes aegypti*. (a) *Eco*RI digests of genomic DNAs probed with the *A. aegypti* rDNA. Ban L - Bangkok strain larval DNA; Ban P - Bangkok pupal DNA; Ban A - Bangkok adult DNA; Lon - London strain larval DNA; Mos - Mos20 cell-line DNA; War - Warwick cell-line DNA; P-N - Porto-Novo strain larval DNA; Cay - Cayenne strain larval DNA; Liv - Liverpool strain larval DNA; Am - American ss strain larval DNA. (b) Restriction maps of the 7.2kb *Eco*RI fragment of *A. aegypti* rDNA (present in clones Aar1, 3, 7 and 9) and of three variant rDNA clones: Aar29, Aar35 and Aav1. The extra *Hin*cII site present in clone Aar9 is shown as an inverted symbol. (■) *Eco*RI; (♦) *Pst*I; (□) *Kpn*I; (▲) *Hin*dIII; (○) *Pvu*II; (◊) *Sac*I; (●) *Hin*cII.

The second approach was to identify DNA sequences which share biochemical and structural properties with previously well defined eukaryotic TGE's. Of particular interest were the class of transposable element known as retrotransposons (RTP's), a characteristic feature of which is their representation in the extrachromosomal fraction of the genome. The generation of linear and/or circular extra-chromosomal DNA copies of RTP integrated forms is a pre-requisite of transposition through a DNA-RNA-DNA pathway. As an initial step towards the identification of *A. aegypti* TGE-like sequences, extrachromosomal DNA was isolated from the MOS20 cell line, digested with *Eco*RI and cloned into the *Eco*RI site of the plasmid vector pBR328. Several clones were randomly picked and used to probe Southern blots of total genomic, *Eco*RI digested *A. aegypti* DNA from a variety of strains. The hybridisation pattern exhibited by one such clone, pX16, which has an insert size of 4.0 kb, is illustrated in Fig. 7. Clearly, this sequence displays intraspecific variation in chromosomal distribution which is a diagnostic feature of TGEs. However, whilst this feature is apparent, the similarity of the hybridisation patterns obtained with MOS20 and

London strain DNA is equally obvious. Furthermore, several bands are common to all of the genomic DNAs analysed, notably the 2.8 kb band. As the MOS20 cell line was derived from London strain larvae, one might expect this similarity - apparent differences in sequence location being a consequence of chromosomal rearrangements, possibly of a transpositional nature, occurring during cell cultivation. The bands common to all DNAs could represent a chromosomal position held by this sequence before division of the species into a variety of strains.

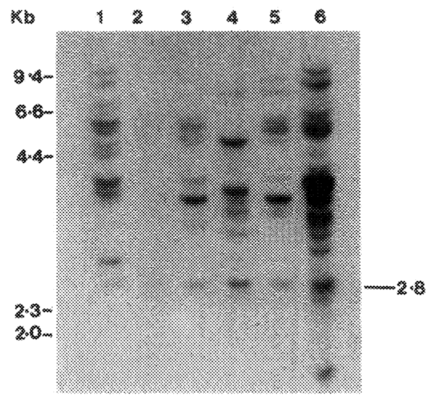


Fig. 7. Southern blot hybridisation of *Eco*RI digested total genomic *Aedes aegypti* DNAs probed with pX16. Lane 1 Cayenne; 2 Thai; 3 London; 4 Liverpool; 5 MOS20; 6 Porto Novo.

Table 2. Partial sequence of pX16 and comparison with reverse transcriptase regions within retroviruses and retrotransposons.

ORIGIN	POSITION[a]	NUCLEIC ACID SEQUENCE[b]	AMINO ACID SEQUENCE[c]
Type 3 oligo'		TATATGGACGACATA	
Aedes aegypti, pX16		CTTGTAATGGACGACATCATAGTG	LVMDDIIV
Retroviruses, MoMuLV	(3258-3282)	AGTACG T T AC GC	QYVDDLLL
Plant viruses, CaMV	(4826-4850)	G GTATG C C C A	VYVDDILV
Drosophila, RTP *copia*	(3682-3706)	T ATATG A T TG GG TA A	LYVDDVVI
.. . 17.6	(3431-3455)	G GTATT A T A	VYLDDIIV
.. . 412	(1413-1437)	TAT T T ATAT C	LYMDDLIV
Yeast, *Ty*	(4681-4705)	T AT CG T T T G T	LFVDDMIL
Human, B-34	(624-651)	GT TGCA T G T A	LFADDMIV

[a] The relevant position of the sequence is numbered according the original publication of the sequence.
[b] Partial DNA sequence of clone pX16 aligned with previously published sequence data from retroviruses, retrotransposons (RTP), and the Type 3 oligonucleotide.
[c] Deduced partial amino acid sequence of pX16 compared with amino acid sequences best conserved among retroviruses and retrotranposons (RTPs).

A further diagnostic feature of TGEs is intraspecific variation in genome copy number. Estimates for the number of copies of pX16 in the genomes of several strains were obtained by comparing densitometric values of pX16 hybridisation to each genomic DNA with those for pX16 self hybridisation. The estimated values range from 30 in the Bangkok strain to 600 in MOS20 DNA, thus confirming the middle repetitive (MR) nature of the pX16 insert

sequence. There was also a comparative 4-fold increase in the copy number of this sequence in MOS20 DNA when compared with that of London DNA. Increases of this magnitude have been reported for several *D. melanogaster* RTPs following passage of embryonic cells in culture (Potter et al., 1979).

Partial DNA sequence data and the deduced amino acid sequence of a portion of pX16 are shown in Table 2 and show considerable homology to the consensus sequence in several retroviral and RTP reverse transcriptases. It should be noted however that the non-conservative amino acid substitution (valine for tyrosine) may have implications for the functionality of this putative enzyme. Further sequence analysis of other members of the pX16 family should help clarify this point.

CsCl/EtBr gradient analysis of MOS20 DNA was used to determine the physical nature of the extrachromosomal DNA from which the pX16 insert was derived. Following gradient fractionation, the refractive index of each aliquot was measured and the EtBr and CsCl removed. Each fraction was EcoRI digested, run through an agarose gel, blotted and probed with radiolabelled pX16 DNA. This experiment clearly illustrated the extrachromosomal origin of this clone. The refractive index of the extrachromosomal fraction corresponded to a density of 1.56 g/ml and as such was indicative of the circular nature of the DNA prior to digestion.

The above properties clearly indicate the mobile nature of pX16. They suggest further a similarity with RTP elements rather than non-viral retroposons. This distinction may be premature although it is based on the findings that the pX16 insert sequence was both isolated from a seemingly circular DNA molecule and has reverse transcriptase (RT) sequence homology. Whilst some non-viral retroposons also show RT homology, to date there is no evidence to suggest their representation as circular molecules in the extrachromosomal DNA. Elucidation of the terminal regions and adjoining host sequences of integrated copies, ie. detection of structural hallmarks such as LTRs and direct host repeats flanking the integrates, should allow classification of this element.

Despite the availability of other gene transfer methods, such as classical plasmid and retroviral techniques, autonomous TGE vector transformation systems may offer several advantages. For instance, the mechanisms of integration employed by TGEs allow exogenous DNA to be inserted into the recipient genome in a disseminated and stable manner. Endogenous TGEs thus appear to offer the prospect of bringing the integration process itself under control and to facilitate the analysis of integration events. This would largely overcome the problems of DNA rearrangement and instability which limit the usefulness, for example, of plasmid vectors (Jacobs et al., 1988; Boeke et al., 1988). Furthermore, it is apparent that the expression of exogenous genes is more adversely affected, following integration, if plasmid vector sequences are linked to the gene under study (Chada et al., 1985). Although retroviral vectors may also offer advantages with respect to experimental control (Brown and Scott, 1987), their major disadvantage is genetic instability, due to high spontaneous mutation rates, aberrant splicing of DNA and lack of proof-reading activity associated with the RNA polymerase and reverse transcriptase reactions. Such genetic instability may well counteract the possible benefits of retroviral systems.

CONCLUSION

The emergence of recombinant DNA technology will soon lead to the cloning of a variety of genes which may have potential in strategies for the suppression of insect populations of the limitation of their vectorial capacity. These will ultimately be transferred into the mosquito germ line by embryo transformation, perhaps following modification or inactivation *in vitro*. Eventually such techniques will provide the raw material to test the drive mechanisms which will be necessary to force manipulated sequences through laboratory and natural populations.

The possible hazards of releasing this type of manipulated organism are difficult to assess in the absence of experimental evidence. What is clear is that this approach has enormous potential which should at least be explored in order to assess and balance the likely benefits against the potential problems.

ACKNOWLEDGMENTS

We thank the Wolfson Foundation, Wellcome Trust, Lister Institute of Preventive Medicine, Medical Research Council and Liverpool University for financial assistance. JMC is a Wellcome Trust Senior Research Fellow in Basic Biomedical Sciences and PE is a Lister Institute Research Fellow.

REFERENCES

Black,W. C. and. Rai, K. S. 1988. Genome evolution in mosquitoes: Intraspecific and interspecific variation in repetitive RNA amounts and organization. Genet. Res. Camb. 51: 185-195.

Boeke, J. D., Xu, H. and Fink, G. R. 1988. A general method for the chromosomal amplification of genes in yeast. Science 239: 280-282.

Brennan, M.D., Rowan, R. G. and Dickinson, W. J. 1984. Introduction of a functional *P* element into the germ line of *Drosophila hawaiiensis*. Cell 38: 147-151.

Britten, R. J., Graham, D. E., Eden, F. C. Painchaud, D. M. and Davidson, E. N. 1976. Analysis of repeating DNA sequences by reassociation. J. Mol. Evol. 9: 1-23.

Brown, A. M. C. and Scott, M. R. D. 1987. pp 109-212 in: "DNA Cloning - A Practical Approach." Vol. III. Glover, D.M., ed. IRL Press, Oxford.

Chada, K., Magram, J., Raphael, K., Radice, G. Lacy, E. and Constantini, F. 1985. Specific expression of a foreign γ-globin gene in erythroid cells of transgenic mice. Nature 314: 377-380.

Chu, G., Hayakawa, H. and Berg, P. 1987. Electroporation for the efficient transfection of mammalian cells with DNA. Nucl. Acid. Res. 15: 1311-1326.

Daniels, S. B., Strausbaugh, L. D. and Armstrong, R. A. 1985. Molecular analysis of *P* element behavior in *Drosophila simulans*. Mol. Gen. Genet. 200: 258-265.

Durbin, J. E. and Fallon, A. M. 1985. Transient expression of the chloramphenicol acetyl transferase gene in cultured mosquito cells. Gene 36: 173-178.

Felgner, P. L., Gadek, T. R., Holm, M., Roman, R., Chan, H. W., Wenz, M., Northrop, J. P., Ringhold, G. M. and Danielson, M. 1987. Lipofection: a highly efficient, lipid mediated DNA transfection procedure. Proc. Natl. Acad. Sci. USA. 84: 7413-7417.

Freshney, R. 1986. Introduction: Principles of sterile technique and cell propagation. pp 1-11 in: "Animal Cell Culture: A Practical Approach." IRL Press, Oxford.

Gale, K. and Crampton, J. M. 1989. The ribosomal genes of the mosquito, *Aedes aegypti*, Eur. J. Biochem. 185: 311-317.

Green,S., Field, J. K., Green, C. D. and Beynon, R. J. 1982. A microcomputer program for analysis of nucleic acid hybridisation data. Nucl. Acid. Res. 10: 1411-1421.

Jacobs, E., Dewerchin, M. and Boeke, J. D. 1988. Retrovirus-like vectors for *Saccharomyces cerevisiae*: Integration of foreign genes controlled by efficient promoters into yeast chromosomal DNA. Gene 67: 259-269.

Knipling, E. F., Laeven, H., Craig, G. B., Pal, R., Kitzmiller, J. B. Smith, C. N. and Brown, A. W. A. 1968. Genetic control of insects of public health importance. Bull. WHO. 38: 421-438.

Lopata, M. A., Cleveland, D. W. and Sollner-Webb, B. 1984. High level transient expression of a chloramphenicol acetyl transferase gene by DEAE-dextran mediated DNA transfection coupled with a dimethyl sulfoxide or glycerol shock treatment. Nucl. Acid. Res. 12: 5707-5717.

McGrane, V., Carlson, J. O., Miller, B. R. and Beatty, B. J. 1988. Microinjection of DNA into *Aedes triseriatus* ova and detection of integration. Amer. J. Trop. Med. Hyg. 39: 502-510.

Miller, L. H., Sakai, R. K., Romans, P., Gwadz, R. W., Kantoff, P. and Caon, H. G. 1987. Stable integration and expression of a bacterial gene in the mosquito *Anopheles gambiae*. Science 237: 779-781.

Morris, A. C., Eggleston, P. and Crampton, J. M. 1989. Genetic transformation of the mosquito, *Aedes aegypti* by micro-injection of DNA. Med. Vet. Ent. 3: 1-7.

O'Brochta, D. A. and Handler, A. M. 1988. Mobility of *P* elements in drosophilids and non drosophillids. Proc. Natl. Acad. Sci. USA 85: 6052-6056.

Potter, S. S., Brorien, W. J., Dunsmuir, P. and Rubin, G. M. 1979. Transposition of elements of the *412*, *copia* and *297* dispersed repeated gene families in *Drosophila*. Cell 17: 429-439.

Spradling, A. C. and Rubin, G. M. 1982. Transposition of cloned *P* elements into *Drosophila* germ line chromosomes. Science 218: 341-347.

Stürchler, D. 1989. How much malaria is there worldwide? Parasitol. Today 5: 39-40.

Steller, H. and Pirrotta, V. 1985. Transposable *P* vector that confers selectable G418 resistance to *Drosophila* larvae. EMBO J. 4: 167-171.

Wigler, M., Siverstein, S., Lee, L. S., Pellicer, A., Cheng, Y. and Axel, R. 1977. Transfer of purified Herpes virus thymidine kinase gene to cultured mouse cells. Cell 11: 223-232.

GENETIC LINKAGE MAPPING WITH RESTRICTION FRAGMENT LENGTH POLYMORPHISMS IN THE TOBACCO BUDWORM, *Heliothis virescens*

C. A. Zraket, J. L. Barth, D. G. Heckel, and A. G. Abbott

Department of Biological Sciences
Clemson University
Clemson, South Carolina 29634

INTRODUCTION

Traditionally, most studies of insect genome structure and expression have been limited to a few "model" organisms with unique biological features that have facilitated classical genetic analysis. Pre-eminent among these are the drosophilid flies, with their small genome size, low chromosome number, high reproductive rates, adaptability to lab culture, ability to withstand inbreeding, and the blessing of polytene chromosomes. This situation is in stark contrast to many lepidopteran species, especially serious pests of agriculture, which have always been *terra incognita* to geneticists. Most lepidoptera have larger genome sizes, much higher chromosome numbers, lower reproductive rates, more exacting culture requirements, and suffer more from inbreeding depression than *Drosophila melanogaster*. Polytene chromosomes are unknown in the order, and only one lepidopteran, the cultivated silkmoth *Bombyx mori,* possesses a detailed linkage map (Tazima 1975). Even this map is of little use in the study of other lepidoptera, because most of the genetic markers are deleterious morphological mutants for which establishment of homologies in other species is problematical. These reasons, among others, explain why genetics has always been one of the weakest links in the application of fundamental biological knowledge to developing biorational methods of lepidopteran pest control.

More recently, however, the use of DNA restriction fragment length polymorphisms (RFLPs) has permitted construction of extensive linkage maps in practically any species of choice. This is a very welcome development, because it means that useful genetic approaches to solving problems are now possible even in species that were manifestly unsuited to the requirements of classical genetical studies. Human genetics provides the most spectacular example, with a highly saturated linkage map based on RFLPs (Donis-Keller et al. 1987) published just eight years after the milestone paper (Botstein et al. 1980) declaring the feasibility of such a project. The wide applicability of linkage mapping with RFLPs is illustrated by a partial list of other species already mapped: tomato (Bernatzky and Tanksley 1986), maize (Helentjaris et al. 1986), lettuce (Landry et al. 1987), and the fungus causing "lettuce downy mildew" (Hulbert et al. 1988).

The main reason for the wide applicability of RFLP technology in genetic mapping is that it allows a simple and relatively direct examination of at least some DNA sequence differences between individuals. Consider any particular segment of DNA at a specific chromosomal location (the "target sequence"). When the DNA is digested to completion with a restriction endonuclease, the resulting cleaved pieces (restriction fragments) of the target sequence may differ in length from individual to individual, due to insertions or deletions between cleavage sites and/or creation or abolition of cleavage sites by base-pair substitutions. When the digested DNA is subjected to agarose gel electrophoresis, fragments are separated according to length. The resulting pattern is transferred by "Southern-blotting" the DNA from the gel to a rigid membrane (Southern 1975), and the positions of the fragments containing the target sequence are revealed by sequence-specific DNA-DNA

Molecular Insect Science, Edited by
H. H. Hagedorn *et al.*, Plenum Press, New York, 1990

hybridization with a labelled probe complementary to the target sequence. Genetic polymorphisms within a population are conveniently visualized by comparing adjacent lanes containing DNA from different individuals, hence the name "restriction fragment length polymorphism."

For construction of saturated genetic maps, marker loci defined by RFLPs have several advantages over classical genetic markers based on morphological mutations or biochemical differences. RFLPs are detectable in all DNA-containing tissues at all life stages, are inherited as codominant nonepistatic Mendelian loci, provide a large number of genetic markers because of the large number of probe/restriction enzyme combinations available, and represent both non-coding and coding portions of the genome. Once identified, RFLP loci can be mapped relative to each other and to any other trait scorable in individuals from which DNA can be isolated.

Our long-term objective is to construct a genetic linkage map in the crop pest *Heliothis virescens* using RFLPs and isozyme markers, and to apply this knowledge to design safer and more reliable means of pest control. *H. virescens,* like most noctuid moths, has a large number (n=31) of chromosomes, which are holocentric and morphologically indistinguishable at metaphase (Chen and Graves 1980). Only a handful of morphological mutants have been described for *Heliothis* species (Bartlett and Roulston 1982). Isozymes have been used as genetic markers in population studies but their genetic basis was not investigated (Sluss and Graham 1979). In spite of this paucity of background genetic information, there is an urgent need for a genetic approach to three problems related to pest control of species of *Heliothis,* arguably the most agriculturally destructive lepidopteran genus worldwide: 1) coping with the rapid evolution of resistance to chemical insecticides (e. g. Leonard et al. 1988), 2) understanding the genetic basis of partial hybrid sterility and its exploitation for autocidal control (Laster, et al., 1988), and 3) preventing or at least slowing the evolution of resistance to genetically engineered toxins from *Bacillus thuringiensis* (Stone et al. 1989).

In this paper we describe our techniques for identifying RFLPs in *Heliothis,* and present preliminary results demonstrating the utility of this molecular approach to genetic map construction.

MATERIALS AND METHODS

Heliothis rearing and crosses

All *Heliothis* stocks and crosses are maintained by methods outlined in Heckel (unpublished). These studies used the PTJ strain of *H. virescens* and the Hs strain of *H. subflexa*. Larvae were individually reared on an artificial diet. Single-pair matings were set up in one-pint paper cans. Full genealogical information was kept for each individual. Full genotypic information was determined for parents after the successful production of offspring. All offspring were reared to adulthood so that adult-stage isozymes could be scored along with RFLPs.

DNA isolation and digestion, and genomic Southern blots

Individual moths were ground in 350 µl of *Heliothis* DNA extraction buffer (0.2M NaCl, 50mM Tris pH 8.0, 1mM DTT, 10mM EDTA, 0.2% SDS) using a motor-driven teflon pestle for 15 to 20 seconds. An additional 350 µl of buffer was added immediately following grinding, and four phenol extractions and one chloroform extraction were performed. Samples were precipitated in ethanol and resuspended in TE (Maniatis et al. 1982, p 448). Restriction enzymes *Eco*RI, *Bam*HI and *Hind*III were used according to the recommendations of the supplier (Promega). Approximately 5 µg of individual moth DNA was digested and electrophoresed on 0.8% agarose gels in TAE (Maniatis et al. 1982, p 454). The gels were blotted (Southern 1975, modified as in Zraket et. al., unpublished) on nylon filters (Hybond-N, Amersham).

Plasmid genomic library construction

Random genomic plasmid clones of *H. virescens* were prepared from phosphatase-treated *Bam*HI fragments of adult DNA and the cloning vector pUC8 (Vieira and Messing

1982). The resulting recombinants were used to transform the bacterial host, *E. coli* strain JM83. Individual colonies with insert-containing plasmids were stored in 25% glycerol at -80°C.

Labelling of probes and hybridization conditions

Approximately 100 ng of cloned insert DNA was labelled using the random priming method of Feinberg et al. (1983). Their procedure was modified by using 20 µCi ^{32}P-dCTP (3000 Ci/mmole) instead of 50 µCi dCTP. Hybridizations were done at 65°C overnight in 6x SSPE, 10x Denhardt's, 1% SDS, 150 µg/ml boiled sonicated salmon sperm DNA (recipes in Maniatis et al. 1982, pp 447-448). Filters were rinsed 4 times in 2x SSC at 65°C for 20 minutes each, followed by a final rinse in 0.3x SSC at 65°C for 20 minutes. Filters were blotted on paper to remove excess fluid, wrapped in Saran-Wrap and exposed to Kodak X-Omat XAR-5 X-ray film with an intensifying screen for seven to eight days (or less for high copy number sequences) at -80°C. Membranes intended for reuse were stripped of probe by gently shaking them in 55% formamide, 2x SSPE, 1% SDS for 45 to 90 minutes at 65°C, then rinsed for one minute at room temperature in 0.1x SSC, 0.1% SDS; and stored in heat-sealable food storage bags.

Prescreening clones to estimate genomic copy number

Isolated plasmid clones were digested with *Bam*HI, electrophoresed on 0.8% agarose gel and blotted on nitrocellulose membranes. The resulting filter was probed with approximately 10^7 cpm/µg of nick translated (Maniatis et al. 1982; Rigby et al. 1977) moth DNA and labelled pUC8. Hybridizations were carried out at 65°C overnight in 3x SSC, 5x Denhardt's, 0.5% SDS, and 100 µg/ml boiled sonicated salmon sperm DNA. The resulting autoradiographs were scored according to methods given in Barth et. al. (unpublished).

RESULTS

Hybridization patterns produced by randomly chosen clones

The first step in building a genetic linkage map using RFLPs is to construct DNA clones complementary to single-copy target sequences in the genome, and then determine which clones reveal polymorphisms in the population. For *H. virescens*, a shotgun genomic DNA library was constructed in the *Bam*HI site of the plasmid vector pUC8. Randomly chosen clones were used to screen individual moth DNA samples for polymorphisms using Southern hybridization analysis. In each case, moth DNA samples were tested using several different restriction enzymes. The results of this random screening were that cloned inserts of three classes of genomic complexity were observed: highly repeated sequences, moderately repeated sequences, and low copy-number sequences (Fig. 1 A, B, and C respectively).

Rapid identification of clones containing single-copy sequences

Clones that are useful for RFLP analysis are ones that detect a small number of fragments and at the same time reveal polymorphisms among individuals (Fig. 1C). Since initial screening of random clones revealed a large number of highly or moderately repeated sequences (Fig. 1 A, B), a method was devised to quickly identify those clones with inserts of low copy number sequences (Barth et al. 1990). Isolated plasmids from randomly selected clones were digested with the restriction enzyme *Bam*HI to release the insert and electrophoresed on a 0.8% agarose gel (Fig. 2A). Subsequently the gel was Southern blotted onto a nitrocellose filter and the filter was hybridized with ^{32}P-labelled genomic moth DNA. Included on the gel were three plasmids known from previous screening to contain sequences that were single copy, moderately repeated, and highly repeated. Any cloned fragment showing a strong hybridization signal (relative to lanes 1, 2, and 3 of Fig. 2B) was scored as a moderate or high repeat sequence (Fig. 2B, lanes 4, 5, 6, 8). Cloned fragments showing no detectable hybridization to genomic DNA after an overnight exposure (Fig. 2B, lane 7) were scored as single or low copy number sequences, and were subsequently screened for polymorphism.

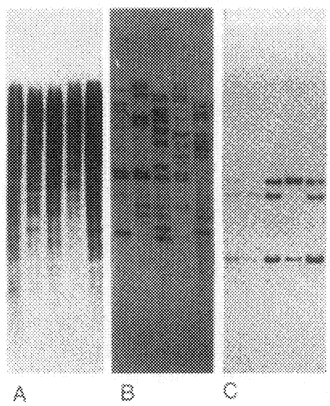


Fig. 1. Hybridization patterns of three classes of genomic DNA probes detected by autoradiography. Southern blots of moth genomic DNA digested with restriction enzymes, *Hind*III or *Eco*RI, were probed with (A) genomic clone 2G6, a highly repetitive DNA sequence, (B) genomic clone 1G1, a moderately repetitive DNA sequence yielding a "fingerprint" pattern, and (C) genomic clone 4F7, a low copy number DNA sequence

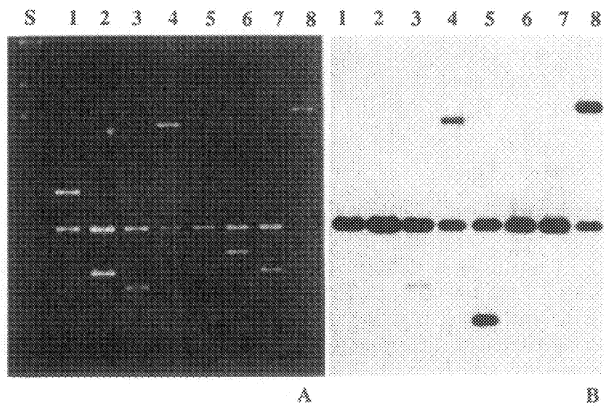


Fig. 2. Rapid selection of clones with single/low copy number DNA sequences. (A) Genomic clones digested with *Bam*HI were electrophoresed on a 0.8% agarose gel and stained with ethidium bromide. Lane S is phage lambda DNA digested with *Hind*III.Lanes 1-3 are standards representing a single/low copy sequence (1), a moderately repeated "fingerprint" sequence (2), and a highly repetitive sequence (3). Lanes 4-8 are genomic clones to be screened. (B) Southern blot hybridization of the same gel probed with labelled *H. virescens* genomic DNA and pUC8. Clones in lanes 4-8 can be scored for repetitive sequence nature based on standards in lanes 1-3.

Identification of polymorphisms

Due to the high chromosome number in *Heliothis*, a large number of polymorphic markers are needed to build a saturated linkage map. To increase the number of loci segregating in a family, interspecific crosses have been employed for linkage mapping (Bernatzky & Tanksley 1986). Theoretically, an interspecific cross should exhibit a higher frequency of RFLPs than an intraspecific cross, since there is a greater genetic divergence between two individuals of different species than between two individuals of the same species. In *Heliothis*, crosses between *H. virescens* males and *H. subflexa* females result in fertile female progeny and sterile male progeny (Miller et al. 1986). The fertile female hybrids can be backcrossed to *H. virescens* males to obtain a potentially highly polymorphic family for linkage analysis.

Cloned DNAs, scored as single copy by the preceding analysis, were tested for their ability to detect polymorphisms in *H. virescens*, *H. subflexa* and their F_1 hybrids (Fig. 3).

Fig. 3. Detection of intra- and interspecific RFLPs in a Southern blot of two *Heliothis* species and their F1 hybrid. DNA of *H. virescens* (lanes 1 and 2), *H. virescens* x *H. subflexa* hybrids (lanes 3 and 4), and *H. subflexa* (lanes 5 and 6) was digested with EcoRI and probed with genomic clone 5E8.

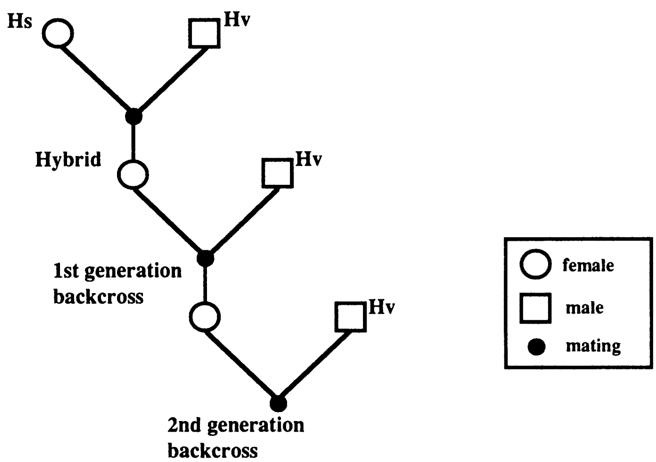


Fig. 4. Lineage of the second generation backcross family used to map RFLP clones. The interspecific cross increases the frequency of RFLPs segregating in a family.

As expected, a high proportion of single copy probes detected interspecific differences, and a somewhat lower fraction also revealed intraspecific differences. Examining hybrids on the same gel served as a check that both parental species were contributing to the hybrid pattern (as would not be the case for mitochondrial DNA or Y-chromosomal DNA). Clones detecting fragment polymorphisms on these filters were then chosen for family segregation studies.

Segregation analysis in backcross families

The inheritance patterns of selected RFLP loci were then examined in a family containing second generation backcross progeny and their first generation backcross mother (Fig. 4). An example of the type of data obtained after hybridization of unique sequence clones to genomic DNA is shown in Fig. 5A and 5B. In each case cloned probes have been hybridized to Southern blots of restriction enzyme digested DNAs of the mother and progeny of the family in Fig. 4. Both probes detect three fragments in the mother: two that cosegregate, and one that segregates independently in the progeny. Additional bands seen in the progeny, but not in the mother are presumed to be inherited from the father (his DNA was not available). In Fig. 5A, there are three paternally derived fragments: two that cosegregate, and one that segregates independently. In Fig. 5B, there are two independently

segregating paternally derived fragments. The progeny are scored for the presence or absence of particular fragments. All four expected genotypes are present in the progeny. Segregation results for each of six RFLP loci were obtained for a total of 22-25 progeny in this second generation backcross family. Results gathered in this segregation analysis and a parallel analysis of isozyme loci are being used to construct a genetic linkage map (Zraket, Heckel and Abbott, unpublished).

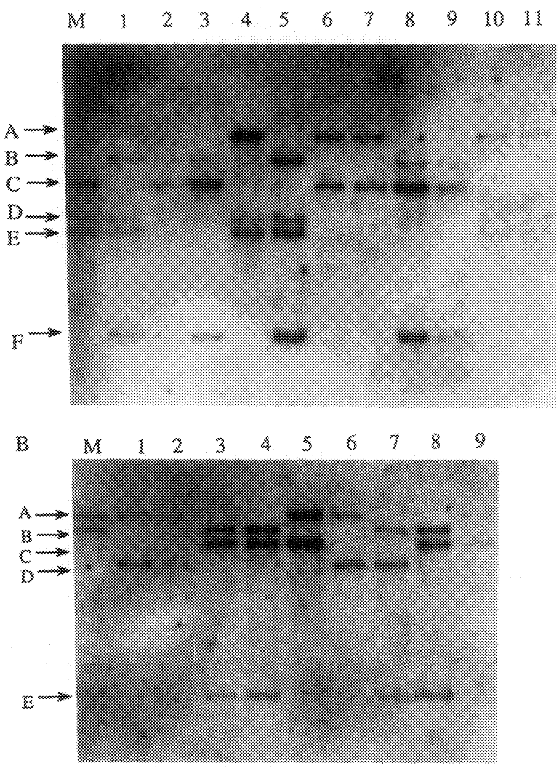


Fig. 5. Autoradiographs illustrating Mendelian segregation of RFLPs in a second generation backcross family. Genomic DNA from mother (lane M) and progeny (numbered lanes) was digested with *Eco*RI and probed with genomic clones 5A9 (in A) and 5B12 (in B).

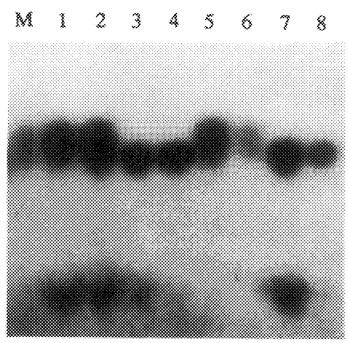


Fig. 6. Starch gel showing GOT-1 (upper bands) and GOT-2 (lower bands) for same family as Fig. 5. Sample origin was at the bottom, and proteins migrated upward toward the anode. GOT-1 genotype *sf* occurs in lanes M, 1, 2, 5, and 6; and *ss* occurs in the other lanes

In order to map isozyme loci relative to RFLP loci, moths were bisected by sagittal section, and DNA extracted from one half and proteins from the other. Fig. 6 shows a starch gel of moth protein extracts from the same progeny depicted in Fig. 5. The gel was stained for the enzyme GOT-1. An interspecific, autosomally-encoded polymorphism exists, with the allele *(f)* from *H. subflexa* encoding a fast-mobility polypeptide, and the *H. virescens* allele *(s)* product having a slower mobility (Heckel 1990b). The active form of this enzyme is a dimer, and so heterozygotes have three bands, corresponding to the fast homodimer, the slow homodimer, and the heterodimer of intermediate mobility. The mother is a *sf* heterozygote and her *H. virescens* mate was a *ss* homozygote, so *ss* and *sf* genotypes are segregating in the progeny. Comparison of the lanes of Figs. 5B and 6 reveals that GOT-1-*sf* cosegregates with 5B12-A, while GOT-1-*ss* cosegregates with 5B12-BE. This suggests that the locus encoding GOT-1 is linked to the RFLP locus 5B12. Analysis of more offspring confirms this hypothesis: no recombinants were found among 22 individuals scored.

DISCUSSION AND CONCLUSIONS

A detailed genetic linkage map based on RFLPs is a powerful tool not yet available for any major insect pest. Yet this goal may be attained relatively soon for *H. virescens*. We have shown that DNA RFLPs are readily detectable in *Heliothis*. We have also shown that these RFLP loci can be scored in families informative for linkage to other RFLPs and to isozyme-encoding loci.

The genome of *H. virescens*, like other moth species (Berry, 1985), appears to contain a high level of repetitive DNA sequences. Therefore, it was necessary to prescreen random genomic clones to identify those carrying unique or low copy number sequences for the mapping analysis. Additional low copy number probes can also be obtained using cDNA clones and heterologous gene clones from other laboratories. Mapping of these clones will expand the genetic marker base and position genes of importance on the genetic map.

We are currently extending these studies by using cDNA, heterologous, and random genomic clones as probes on DNA samples from a first generation backcross family. In this family the *H. virescens* x *H. subflexa* hybrid mother, *H. virescens* father, and the progeny are available for the analysis. This should provide an even greater degree of polymorphism than the second generation backcross family discussed in this communication.

A saturated linkage map is important for determining genome organization and for potential gene manipulation. RFLP markers can be linked to traits or genes of interest and these genes followed in crosses or in populations in the field. Furthermore, if the map is sufficiently saturated, RFLP loci can be used to initiate a chromosomal "walk" to a gene of interest so that it can be cloned.

Currently, one of the major traits of concern in *Heliothis* populations is the development of insecticide resistance. Having genetic markers for such traits will facilitate more detailed physiological studies on the mechanisms of resistance, and allow more accurate estimation of the level of resistance in field populations. This knowledge in turn will be useful in insecticide resistance management, in developing countermeasures to resistance, and in allowing effective, alternative control strategies to be developed. Thus linkage mapping using RFLPs has the potential to transform the genetical approach from one of the weak links in our knowledge of insect pests, to an integral component in the design of more effective, less environmentally hazardous, methods of pest control.

ACKNOWLEDGMENTS

This work was supported in part by USDA Competitive Grant No. 87-CRCR-1-2506 and USDA-ARS Specific Cooperative Agreement No. 58-32U4-7-078. We thank T. M. Brown for introducing us to *Heliothis* and for his continued encouragement of this project, Candace A. Cummings for technical assistance, and W. Klassen, R. L. Ridgway, and M. D. Huettel of USDA-ARS for their interest and support.

REFERENCES

Bartlett, A. C. and Roulston, J. R. 1982. The identification and use of genetic markers in population dynamics and control studies in *Heliothis*. pp. 75-85 in: "Proceedings of the International Workshop on *Heliothis* Management." ICRISAT. Patancheru, A. P., India.

Bernatzky, R. and Tanksley, S. D. 1986 Toward a saturated linkage map in tomato based on isozyme and random cDNA sequences. Genetics 112: 887-898.

Berry, S. J. 1985. Insect nucleic acids. pp. 219-253 in: "Comprehensive Insect Physiology, Biochemistry, and Pharmacology." Vol. 10. Kerkut, G. A. and Gilbert, L. I. eds. Pergamon Press, Oxford.

Botstein, D., White, R. L. , Skolnick, M., and Davis, R. W. 1980. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. Am. J. Human Genetics 32: 314-331.

Chen, G. T., Graves, J. B. 1970. Spermatogenesis of the tobacco budworm. Ann. Entomol. Soc. America 63: 1095.

Donis-Keller, H., Green, P., Helms, C., Cartinhour, S., Weiffenbach, B. et al. 1987. A genetic linkage map of the human genome. Cell 51: 319-337.

Feinberg, A. P. and Vogelstein, B. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 132: 6-13.

Helentjaris, T., Slocum, M., Wright, S., Schaefer, A., and Nienhuis, J. 1986. Construction of genetic linkage maps in maize and tomato using restriction fragment length polymorphisms. Theor. Appl. Genet. 72: 761-769.

Hulbert, S. H., Ilott, T. W., Legg, E. J., Lincoln, S. E., Lander, E. S. and Michelmore, R. W. 1988. Genetic analysis of the fungus, *Bremia lactucae,* using restriction fragment length polymorphisms. Genetics 120: 947-958.

Landry, B. S., Kesseli, R. V., Farrara, B., and Michelmore, R. W. 1987. A genetic map of lettuce (*Lactuca sativa*) with restriction fragment length polymorphism, isozyme, disease resistance and morphological markers. Genetics 116: 331-337.

Laster, M. L., Carpenter, J. E., Huettel, M. D., Miller, S. G., Proshold, F. I., Roush, R. T., Schneider, J. C., and Sparks, A. N.. 1988. Autocidal systems for *Heliothis* control. pp. 74-103 in: "Theory and Tactics of *Heliothis* Population Management: III. Emerging Control Tactics and Techniques." G. A. Herzog, et al., eds. Southern Cooperative Series, Stillwater, Oklahoma.

Leonard, B. R., Graves, J. B., Sparks, T. C., and Pavloff, A. M.. 1988. Variation in resistance of field populations of tobacco budworm and bollworm to selected insecticides. J. Econ. Entomol. 81: 1521-1528.

Maniatis, T., Fritsch, E. F., and Sambrook, J. 1982. "Molecular Cloning: a Laboratory Manual." Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Miller, S. G., Huettel, M. D., Davis, M. B., Weber, E. H., and Weber, L. A. 1986. Male sterility in *Heliothis virescens* x *H. subflexa* backcross hybrids. Mol. Gen. Genet. 203: 451-461.

Rigby, P. W. J., Kiekmann, M., Rodes, C., and Berg, P. 1977. Labelling DNA to high specific activity by nick translation with DNA polymerase I. J. Mol. Biol. 113: 237-251.

Sluss, T. P. and Graham, H. M. 1979. Allozyme variation in natural populations of *Heliothis virescens* in North America. Ann. Entomol. Soc. Am. 72: 317-322.

Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98: 503-517.

Stone, T. B, Sims, S. R., and Marrone, P. G. 1989. Selection of tobacco budworm for resistance to a genetically engineered *Pseudomonas fluorescens* containing the delta-endotoxin of *Bacillus thuringiensis* subsp. *kurstaki*. J. Invert. Pathology 53: 228-234.

Tazima, Y., Doira, H., and Akai, H. 1975. The domesticated silkmoth, *Bombyx mori*. pp. 63-124 in: "Handbook of Genetics. vol. 3: Invertebrates of Genetic Interest." King, R. C. ed. Plenum Press, New York.

Vieira, J. and Messing, J. 1982. The pUC plasmids, and M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene 19: 259-271.

HOMEOTIC GENES OF THE RED FLOUR BEETLE, *Tribolium castaneum*

R.W. Beeman[1], S.J. Brown[2], J.J. Stuart[1], and R.E. Denell[2]

[1]U.S. Grain Marketing Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Manhattan, KS 66502
[2]Division of Biology, Kansas State University, Manhattan, KS 66506

INTRODUCTION

The powerful combination of genetic, developmental and molecular methodologies available for *Drosophila melanogaster* has made it the premier insect experimental system for studies in many areas. Although there is no doubt that *D. melanogaster* should continue to be the first choice for investigations of many fundamental phenomena, there is also a strong rationale in many contexts for a comparative approach utilizing other insect systems. For example, *D. melanogaster* is highly specialized with respect to the events of segmentation and many aspects of anterior development.

We have been working to develop the red flour beetle, *Tribolium castaneum*, as another insect system allowing integrated developmental genetic and molecular analysis; our studies are initially focused on the genetic control of segmentation and segmental identity. The main rationale for choosing this insect is the possibility of high resolution genetic analysis (Beeman et al., 1989). We have shown further that *T. castaneum* has a genomic size of 2.1×10^8 bp (slightly larger than *D. melanogaster*) and a long period interspersion pattern of repeated DNA, characteristics which make it a convenient organism for molecular genetic studies (Brown et al., in press). Moreover, *T. castaneum* is a pest of stored grain and grain products of global significance, and also has the potential to serve as an important model system for harmful Coleoptera in general. These advantages recommend this beetle for many types of studies.

SEGMENTATION IN *Drosophila melanogaster* AND *Tribolium castaneum*

We have followed embryonic development in whole mounts to confirm that events in *T. castaneum* closely resemble its better described sibling species, *Tribolium confusum* (see Sokoloff, 1972). After formation of the cellular blastoderm, a ventral germ band appears. The early germ band rapidly becomes relatively complex in three dimensions as the extraembryonic layer forms and the mid-ventral gastral furrow invaginates to define the presumptive mesoderm. Segmentation as indicated by superficial epidermal grooves occurs rapidly through the gnathal and thoracic metameres, and then more slowly as the germ band extends and ten abdominal segments are progressively delineated. After separation of the ectoderm into the neurogenic region and the presumptive epidermis, evaginations representing appendage primordia appear on the antennal, gnathal, thoracic and first abdominal segments. Thereafter, the stomodeum and the proctodeum are formed; the latter initially occupies a mid-dorsal position at the posterior terminus of the germ band. As development continues the appendages grow and become better defined, and the proctodeum moves back toward the posterior pole as the germ band contracts. Thereafter, the germ band broadens laterally to accomplish dorsal closure and cuticle deposition occurs.

Segmentation in *T. castaneum* is relatively primitive compared to the advanced embryonic behavior of *D. melanogaster*. The entire latero-ventral surface of the D. *melano-*

Molecular Insect Science, Edited by
H. H. Hagedorn *et al.*, Plenum Press, New York, 1990

gaster cellular blastoderm initially represents the germ band, which undergoes extension, and then segments simultaneously along its entire length. This behavior is termed "long germ" development, as opposed to the more primitive "short" or "intermediate germ" behavior typical of most insects (Sander, 1976). Metamerization is signaled by the occurrence of grooves in the ectoderm and mesoderm. Martinez-Arias and Lawrence (1985) have argued that in *D. melanogaster* these grooves are parasegmental (i.e., at the anterior/-posterior compartment boundary) rather than segmental. Later in development, the germ band contracts to bring the proctodeum into a posterior position.

The process of segmentation in *D. melanogaster* is controlled by complex interactions between many genes, most of which encode transcription factors (Akam, 1987; Ingham, 1988). The spatial pattern of expression of many of these genes shows dynamic changes during the developmental events leading up to overt metamerization. One very interesting question is the extent to which the evolution of long germ behavior is reflected in the roles and patterns of expression of the genes identified and studied in *D. melanogaster*.

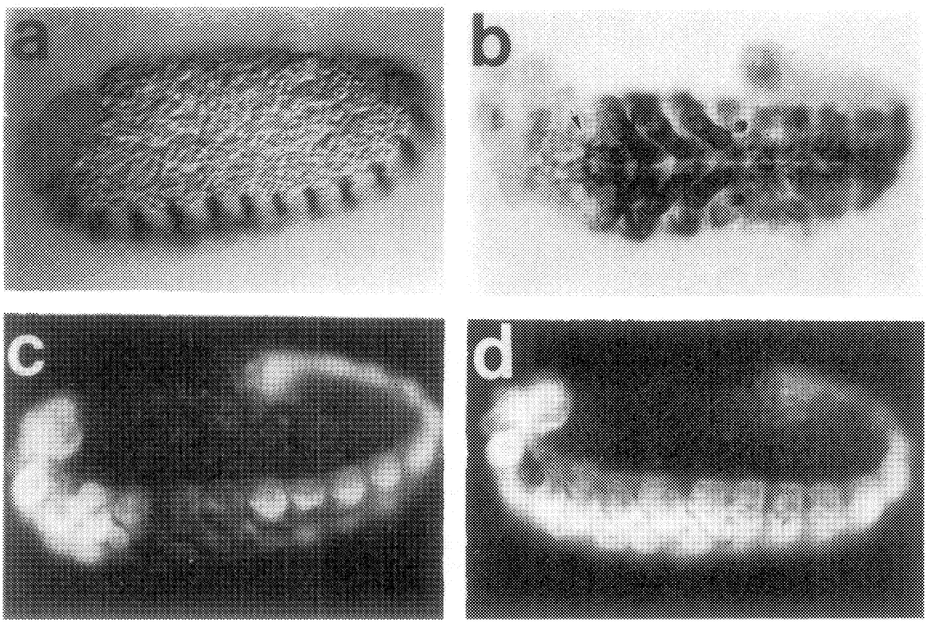


Figure 1: A: lateral view of a wildtype *Tribolium castaneum* embryo immunostained with an antibody against the *Drosophila melanogaster engrailed* protein and examined by differential interference contrast microscopy. B: ventral view of a wildtype *T. castaneum* embryo immunostained with an antibody against the *D. melanogaster Antennapedia* protein and examined by bright field microscopy. The arrowhead indicates immunostained nuclei in the posterior labial ganglion. C and D: lateral views of a wildtype and a ptl^{D60} homozygous embryo, respectively, immunostained with *anti-Antennapedia*, counterstained with Hoechst 33258, and examined by fluorescence microscopy. In all panels, anterior is to the left.

One of these genes, *engrailed* (*en*), provides an especially useful molecular marker (Akam, 1987). Developmental genetic studies indicated that the normal function of this gene is required to define the posterior compartment of each segment, and studies utilizing *in situ* hybridization or antibodies against the encoded protein show that segmented embryos express the gene in domains representing the posterior compartment of the epidermis of each segment in the posterior head and body. Later in development, *en* is also expressed in the posterior portion of the ganglion associated with each of these segments. The encoded protein is nuclear, includes a homeo domain, and functions as a transcriptional regulator (Desplan et al., 1988). Interestingly, *en* expression does not just appear in its final distribution, but develops in a complex manner dependent on interactions with other segmentation genes (DiNardo and O'Farrell, 1987; Karr et al., 1989).

We have immunostained *T. castaneum* embryo whole mounts with 4D9, a monoclonal antibody which recognizes an epitope of the *en* protein (Patel et al., 1989). In the epidermis and central nervous system of fully segmented embryos, we observe a pattern similar to that of *D. melanogaster*. That is, each segment of the embryo shown in Figure 1A shows intense staining in the posterior portion of the ganglion and a more weakly staining epidermal stripe. The restriction of this *T. castaneum* antigen to the posterior region of each segment argues that it is encoded by a gene homologous to *en*. It is clear that after the gnathal and thoracic metameres have been segmented, a stripe of *en* expression is present immediately anterior to each of the superficial transverse grooves which delineate them. This observation indicates that these grooves are segmental rather than parasegmental, contradicting the assertion of Lawrence (1988) that the occurrence of parasegmental grooves as the first evidence of metamerization will be the general rule among insects. As the germ band extends well ahead of the progressive segmentation of the abdomen, an *en* stripe precedes the formation of each new segmental groove. These results imply that although *engrailed* has maintained a similar final pattern of expression and presumably a similar role during insect evolution, the establishment of that pattern can vary strongly depending on the mode of germ band formation and presumably on interactions with the other genes involved in that process. Our independent results are very similar to those described by Patel et al. (1989) for the grasshopper, *Schistocerca americana*.

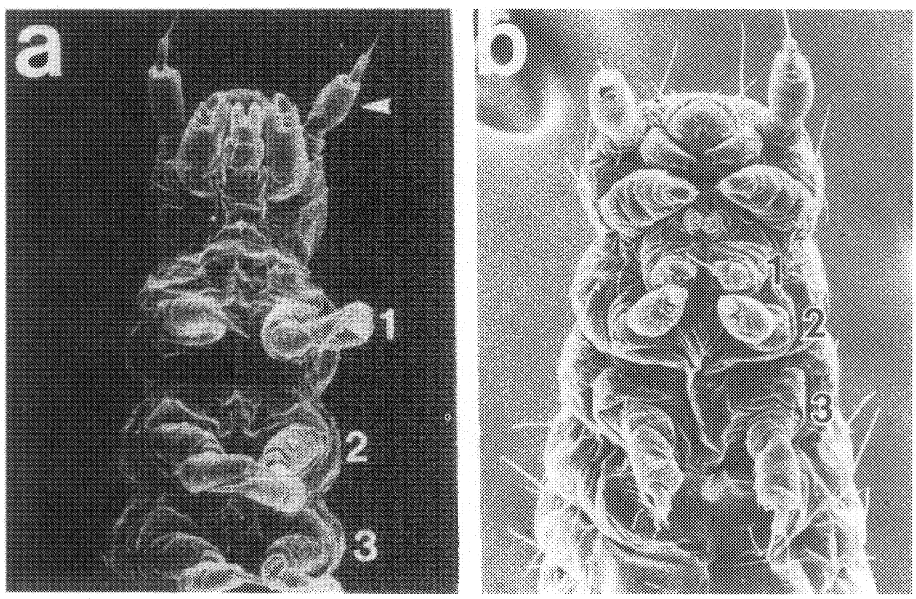


Figure 2: Ventral views of a wildtype larva (A) and a ptl^{D60} homozygous larva (B) examined by scanning electron microscopy. Numbers indicate the appendages (legs in A and antennae in B) on each of the three thoracic segments. An arrowhead indicates a normal antenna.

HOMEOTIC GENES AND GENE COMPLEXES

Studies with *D. melanogaster* have shown that the developmental fates of cells in each metamere are controlled by the homeotic genes. The genes which assign fates in the posterior embryo form a cluster called the bithorax complex (see Duncan, 1987). Genetic studies have recognized three classes of lethal mutations within the complex which correspond to three transcription units: *Ultrabithorax*, *abdominal-A*, and *Abdominal-B*. These appear to be the only protein-coding transcription units in the complex, but they are associated with regulatory regions of great size and complexity.

A second gene cluster termed the Antennapedia complex includes the homeotic genes

Antennapedia, *Sex combs reduced*, *Deformed*, *proboscipedia*, and *labial* which control determinative decisions in the head and thorax (see Kaufman and Olsen, 1990). However, the Antennapedia complex differs from the bithorax complex in having other genes which are not ostensibly homeotic in nature, such as the well-studied *fushi tarazu*, *zerknullt*, and *bicoid* loci.

Previously, one of us (Beeman, 1987) mapped the extant homeotic mutations in *T. castaneum*, and showed that there is a single complex of homeotic genes representing the Antennapedia and bithorax complexes in juxtaposition. We believe that this organization is likely to be the primitive condition among insects. Taken together, evidence from *D. melanogaster* and *T. castaneum* suggests that the ancestral complex showed "colinearity" (Lewis 1978); that is, the order of genes along the chromosome parallels their function along the anterior/posterior axis of the embryo. Recent evidence has shown that vertebrates have complexes similar in organization to that of the *T. castaneum* homeotic gene complex (Douboule and Doll, 1989; Graham et al., 1989), indicating that the origin of such clusters is very ancient.

We have used mutagenesis screens to isolate many point mutations and chromosomal rearrangements in the homeotic gene complex, some of which serve as effective balancers for the region (Beeman et al., 1989). We have thus far isolated approximately 70 mutations recognized because they fail to complement the original recessive homeotic variants and/or because of dominant effects. In most cases, we have also studied the mutant phenotype at all stages of the life cycle. As summarized in Table 1 (Beeman et al., 1989, and unpublished results), we have thus far identified six homeotic complementation units which (based on phenotypic similarities and some other evidence presented below) we hypothesize are homologous to the six *D. melanogaster* homeotic genes indicated. Here we will concentrate on the two loci that have been most extensively studied.

THE *T. castaneum* *PROTHORAXLESS* GENE

The original *prothoraxless* (*ptl*) mutation arose spontaneously, and is a largely recessive variant causing a reduction in the size of the first thoracic segment (T1) of the adult which is interpreted as a partial transformation to resemble the labium. Recently we have characterized five additional dominant alleles associated with reductions of T1 in heterozygotes. Since each dominant *ptl* allele is complemented by a duplication, we believe that it is a haplo-insufficient locus.

Homozygotes for apparent null alleles die as late embryos or first instar larvae. Ventrally, these individuals show a dramatic transformation of legs to antennae as well as rudimentary labial palps which are closely opposed to the T1 appendages (Figure 2B; compare to the wildtype larva pictured in 2A). Dorsally the thorax is strongly reduced (presumably also a reflection of a partial transformation to antenna), but shows a reiteration of some elements of the labium.

Two aspects of the *ptl* phenotype are similar to those of *Antennapedia* mutants. In *Antp*$^-$ homozygotes (Martinez-Arias, 1986), parasegments 4 and 5 are partially transformed to resemble PS3 (posterior labium/anterior T1), an effect which at least parallels the reiteration of the labium in *ptl* homozygotes. Moreover, in adults *Antp*$^-$ clones in ventral T2 show a transformation to antenna (Struhl, 1981; Abbott and Kaufman, 1986). As one way to examine the possible relationship between these genes, we tested two monoclonal antibodies raised against a protein encoded by the *D. melanogaster Antp* gene (Glicksman and Brower, 1988) to determine whether they recognized a conserved epitope expressed by T. castaneum embryos. Both 4C3 and 8C11 gave similar patterns; the former was utilized for most studies described below.

Young embryos show no immunostaining until the germ band has formed and extended to just wrap around the posterior pole to reach the dorsal surface; at this stage, only the head and thoracic segments are defined by intersegmental grooves. Although the head is negative, thoracic and abdominal portions of the germ band, including both the embryo proper and the overlying extraembryonic layer, have immunopositive nuclei. As development proceeds, nuclei in the extending germ band are also positive. Later in development, as the central nervous system forms, the ganglia show nuclear immunostaining as well (Figure 1B). Similar to *D. melanogaster*, the intensity of staining varies considerably among the nuclei within each ganglion; casual inspection indicates that the spatial pattern of darkly stained nuclei is bilaterally symmetrical and reiterated within each ganglion. Unlike staining in the

epidermis, expression in the CNS extends into the posterior labial ganglion. Later in development, additional immunostaining appears which is much more broadly distributed and is not nuclear in nature. The proctodeum, pharynx and pleuropodia (putative glands on the first abdominal segment) are particularly intensely stained, but the epidermis or cuticle of all segments (including the head) is more lightly stained.

We have used 4C3 to examine embryos homozygous for dominant *ptl* alleles. Several types of evidence convince us that such homozygotes are immunonegative with respect to the nuclear staining normally observed in the epidermis and CNS, although they still show the more general staining observed in older embryos. At a developmental stage when immunostaining is normally restricted to the epidermis and ganglia of body segments (and the posterior ganglion of the labium), homozygotes stained with the DNA-specific dye Hoechst 33258 and viewed by fluorescence microscopy can be identified by leg abnormalities and the absence of a T2 spiracle. Such embryos (Figure 1D) are invariably immunonegative. The wildtype embryo pictured in Figure 1C shows occlusion of nuclear fluorescence in the thorax because of immunostaining (the staining pattern is not as striking as in 1B because we have not cleared these Hoechst-stained embryos). These and other observations lead us to the interpretation that: 1) *ptl* is the homolog of the *Antp* gene, and 2) the antibodies utilized recognize both the *ptl* gene product, which shows a nuclear distribution in the epidermis, CNS, and some other tissues, and some other antigen which is unrelated to the *ptl* gene.

The conclusion that *ptl* and *Antp* are homologous raises many interesting points. In a broad sense, the distribution of the respective proteins encoded by these genes, at least as detected by immunological studies, is rather similar. *Antp* protein distribution depends on each of the *Antp* promoters in a pattern that is complex and continuously changing during embryogenesis (Carroll et al., 1988; R. Garber, personal communication). Overall, the protein is expressed in the epidermis of all parasegments from PS4 to 14, as well as the extraembryonic amnioserosa. In the ventral nerve cord, expression extends into the posterior labium. Except for the lack of parasegmental regulation in the epidermis, the same antibody detects a very similar overall distribution in *T. castaneum*. However, space precludes a discussion of the many differences in the details of expression in the two organisms. Even more striking are the differences in the mutant phenotypes already summarized above. Clearly, if our interpretation of homology is correct, these genes have evolved considerable differences in the determined states established by their actions. It will be fascinating to study how evolution has modified the function of this regulatory gene, presumably through changes in its target genes.

THE *T. castaneum ABDOMINAL* GENE

The *Abdominal* gene was originally recognized via two recessive hypomorphic alleles (Beeman, 1987). Adults homozygous for *missing abdominal sternite* (*mas*) and *pointed abdominal sternite* (*pas*) show anterior transformations of the third and fourth abdominal segments (A3 and A4), respectively. Further mutagenesis has generated over 20 dominant variants associated with four phenotypic classes: *Abdominal* (*A*), *Extra sclerite* (*Es*), *Socketless* (*Skl*), and *Miscaudal sclerotization* (*Mcs*). *A* variants are the most frequent, and we interpreted their dominant, posterior transformation of A6 as the haplo-insufficient effect of loss-of-function mutations (Beeman et al., 1989). *A* mutations also fail to complement *mas* and *pas*. The other classes all include transformations opposite in direction to those of *A* variants, and were interpreted as gain-of-function variants of the same gene. We have since confirmed this hypothesis by showing that *Es, Skl*, and *Mcs* variants can all be mutated by ionizing radiation at rates characteristic of forward mutation to eliminate the putative gain-of-function effects; in each case, the revertant expresses the *A* phenotype and fails to complement other mutant alleles at that gene.

In the bithorax complex, loss-of-function mutations cause anteriorily-directed transformations and gain-of-function mutations result in posteriorly-directed changes. Lewis (1963, 1978) has argued that insects arose from ancestors in which body segments were essentially identical, and that new bithorax complex functions evolved to confer more and more posterior identities with respect to a thoracic "ground state". Thus, although the observation that loss-of-function *A* mutations cause anteriorly-directed transformations of the anterior adult abdomen was expected, the posteriorly-directed changes of the posterior abdomen were quite surprising.

We have since studied the embryonic lethal syndrome of *A* homozygotes, and we see quite a different picture: abdominal segments 2 to 8 are transformed to resemble the first abdominal segment. During embryogenesis, the first segment normally develops appendage-like buds similar to, but smaller than, those which give rise to the thoracic legs. Later in development, these buds are retracted and form the pleuropodia. Homozygotes of *A* have buds on every abdominal segment through the 8th which develop into pleuropodia.

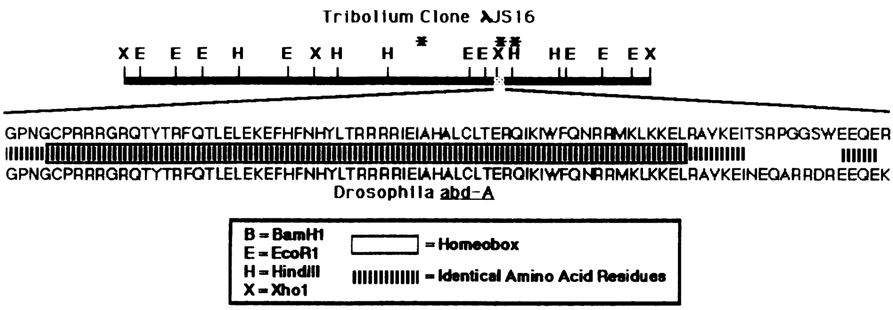


Figure 3: A restriction map of *Tribolium castaneum* genomic insert λJS16, as well as the predicted amino acid sequence of the homeo box and flanking regions compared to that of the *Drosophila melanogaster abdominal-A* gene (W. Bender, personal communication). The restriction fragments indicated by asterisks identify restriction fragment length polymorphisms as discussed in the text.

The phenotype of *A* mutations strongly argues for the interpretation that this gene is homologous to the *abdominal-A* (*abd-A*) gene of *D. melanogaster*. In *abd-A⁻* embryos PS7 through PS9 are transformed toward PS6 (posterior T3/anterior A1), whereas PS10-13 are more weakly transformed. However, the posterior abdomen is more strongly affected in *Abd-B* mutants (Duncan, 1987). The observation that *A* mutants show a strong transformation of all abdominal segments through the 8th is interesting in that Akam et al. (1988) have argued that the domain of *Abd-B* in primitive insects is likely to be in segments posterior to the 8th, and that its function in more anterior segments in *D. melanogaster* is a derived characteristic. Restated, they would argue that the primitive case is one in which *abd-A* has a strong phenotypic effect throughout the abdomen. That idea is consistent with our results and the phenotype of a presumed *abd-A* homolog in the silk moth (Tazima, 1964).

Using a *D. melanogaster* homeo box probe, we have isolated the *T. castaneum* homologs of a number of homeotic genes. One set of overlapping genomic clones includes a region with clear homology to *abd-A*. The predicted amino acid sequence within a homeo box and in flanking regions is entirely conserved with respect to that of *abd-A* (Figure 3). With respect to the restriction map also presented for the region, the *Eco*R1/-*Hin*dIII fragment indicated by a star identifies a restriction length polymorphism differing between the Georgia background on which many homeotic gene complex variants were induced and a balancer chromosome derived from the LAB-S strain and bearing the mutation *Stumpy*. Recombinational analysis shows that this restriction length polymorph-ism cosegregates with the homeotic gene complex. In addition, we have shown by restriction mapping that the ionizing radiation-induced mutation A^{12} is associated with a chromosomal rearrangement breakpoint in the ~400 bp *Xho*I/*Hin*dIII fragment marked by a double star. This breakpoint is in or near the homeo box. High resolution recombination studies show that this restriction length polymorphism maps within the homeotic gene complex at the location of the *Abdominal* gene. These results provide strong evidence that *Abdominal* is homologous to *abdominal-A*.

PERSPECTIVES

Results summarized here argue strongly that *T. castaneum* offers a powerful insect

Table 1. Phenotypes Associated with HOM-C Loss-of-Function Mutant Alleles

LOCUS	PUTATIVE DROSOPHILA HOMOLOG	NUMBER ALLELES	HOMOZYGOUS LETHAL PERIOD	ADULT PHENOTYPE[1]	EMBRYONIC OR LARVAL PHENOTYPE
maxillopedia (mxp)	pb	20	Larval	MX, LA->leg	MX, LA->leg
Cephalothorax (Cx)	Scr	7	Larval	LA->T1, T1->T2	LA->ANT, cephalothorax[2]
prothoraxless (ptl)	Antp	6	Emb/Lar	T1->LA	Ventral T1, 2, 3->ANT[3]
Ultrathorax 9Utx)	Ubx	3	Embryo[4]	T3->T2	A1->T3
Abdominal (A)	abd-A	29	Embryo	A3->A2, A4->A3, A6->A7, A7->A8	A2-A8->A1
extra urogomphi (eu)	Abe-B	1	None[5]	None	A10->A9

[1]Abbreviations: antenna (ANT), Maxillary (MX), Labial (LA), Prothorax (T1), Mesothorax (T2), Metqthorax (T3), Abdominal segments (A1-A10)

[2]There are actually two lethal phenotypes associated with Cx homozygotes, and we don't as yet know which is the loss-of-function phenotype. In the phenotype indicated in the Table, labial palps are transformed to antennae and T1 is cephalized, so that the apparent head is actually a cephalothorax consisting on the head + T1. Other genotypes show transformations of labium-to-maxilla and the appearance of a dorsal supernumerary T1 segment juxtaposed between the head and a normal T1.

[3]Other aspects of the *ptl* lethal syndrome are described in the text.

[4]We have relatively few alleles of this gene, and have not yet performed experiments which will critically assess which alleles, if any, display a null phenotype.

[5]We have only a single, spontaneous recessive allele of eu, and suspect from recovering a number of sterile individuals with an eu-like phenotype that null mutations are male and female sterile in heterozygous condition. Thus, we cannot state with any confidence that this is the null phenotype.

system for integrated developmental, genetic and molecular studies, and provide a solid foundation for further analysis of the HOM-C. Evidence cited for *prothoraxless* and *Abdominal* support the hypothesis that the single homeotic gene complex in *Tribolium* and the combined pair of homeotic gene complexes in *D. melanogaster* are homologous, and indicate that the genes identified by mutation do indeed correspond to the ones that can be molecularly cloned with heterologous probes. One important future objective will be to use available balancers and deficiencies to saturate the complex with mutations in order to detect all genetic functions present; such a goal is presently inconceivable in any other organism outside of the Diptera. Moreover, in addition to *Abdominal* we have cloned the homologs of the *D. melanogaster Antennapedia, Deformed*, and *Ultrabithorax* genes; these and others will allow us to detect the patterns of expression of the *T. castaneum* homeotic genes during development.

ACKNOWLEDGMENTS

This research was supported in part by NSF grant DCB-8702728, American Cancer Society grant NP-663, and NASA grant NAGW-1197 to BioServe Space Technologies. SJB was supported as a Wesley Foundation Scholar in Cancer Research, and JJS as a USDA Postdoctoral Fellow. We thank Drs. R.L. Garber and T.C. Kaufman for probes, Drs. D.L. Brower and T.B. Kornberg for antibodies, Drs. W. Bender and R.L. Garber for sharing information prior to publication, and Katherine Hummels and Susan Haas for technical assistance. Part of this work was performed by RED in the laboratory of Dr. R.L. Garber, for which he is most appreciative.

REFERENCES

Abbott, M. K. and Kaufman, T. C. 1986. The relationship between the functional complexity and the molecular organization of the *Antennapedia* locus of *Drosophila melanogaster*. Genetics 114: 919-942.

Akam, M. 1987. The molecular basis for metameric pattern in the *Drosophila* embryo. Development. 101: 1-22.

Akam, I., Dawson, I., and Tear, G. 1988. Homeotic genes and the control of segment diversity. Development 104 (suppl.): 123-133.

Beeman, R. W. 1987. A homoeotic gene cluster in the red flour beetle. Nature 327: 247-249.

Beeman, R. W., Stuart, J. J., Haas, M. S., and Denell, R. E. 1989. Genetic analysis of the homeotic gene complex (HOM-C) in the beetle *Tribolium castaneum*. Dev. Biol. 133: 196-209.

Brown, S. J., Henry, J. K., Black, W. C. IV, and Denell, R. E. Molecular genetic manipulation of the red flour beetle: Genome organization and cloning of a ribosomal protein gene. Insect Biochem. (in press).

Carroll, S. B., DiNardo, S., O'Farrell, P. H., White, R. A. H. and Scott, M. P. 1988. Temporal and spatial relationships between segmentation and homeotic gene expression in *Drosophila* embryos: Distributions of the *fushi tarazu, engrailed, Sex combs reduced, Antennapedia*, and *Ultrabithorax* proteins. Genes Dev. 2: 350-360.

Desplan, C., Theis, J. and O'Farrell, P. H. 1988. The sequence specificity of homeodomain-DNA interaction. Cell 75: 1081-1090.

DiNardo, S. and O'Farrell, P. H. 1987. Establishment and refinement of segmental pattern in the Drosophila embryo: spatial control of *engrailed* expression by pair-rule genes. Genes Dev. 1: 1212-1225.

Duboule, D. and Dollé, P. 1989. The structural and functional organization of the murine HOX gene family resembles that of *Drosophila* homeotic genes. Embo. J. 8: 1497-1505.

Duncan, I. 1987. The Bithorax complex. Ann. Rev. Genet. 21: 285-319.

Glicksman, M. A. and Brower, D. L. 1988. Misregulation of homeotic gene expression in *Drosophila* larvae resulting from mutations at the *extra sex combs* locus. Dev. Biol. 126: 219-229.

Graham, A., Papalopulu, N., and Krumlauf, R. 1989. The murine and *Drosophila* homeobox gene complexes have common features of organization and expression. Cell 57: 367-378.
Ingham, P. W. 1988. The molecular genetics of embryonic pattern formation in *Drosophila*. Nature 335: 25-34.
Karr, T. L., Weir, M. P., Ali, Z. and Kornberg, T. 1989. Patterns of *engrailed* protein in early *Drosophila* embryos. Development 105: 605-612.
Kaufman, T. C. and Olsen, G. 1990. The homeotic genes of the *Antennapedia* gene complex of *Drosophila melanogaster*. Amer. Nat. (in press).
Lawrence, P. A. 1988. The present status of the parasegment. Development 104(suppl.): 61-64.
Lewis, E. B. 1963. Genes and developmental pathways. Am. Zool. 3: 33-56.
Lewis, E. B. 1978. A gene complex controlling segmentation in *Drosophila*. Nature 276: 565-570.
Martinez-Arias, A. 1986. The *Antennapedia* gene is required and expressed in parasegments 4 and 5 of the *Drosophila* embryo. EMBO J. 5: 135-141.
Martinez-Arias, A. and Lawrence, P. A. 1985. Parasegments and compartments in the *Drosophila* embryo. Nature 313: 639-642.
Patel, N. H., Kornberg, T. B. and Goodman, C. S. 1989. Expression of *engrailed* during segmentation in grasshopper and crayfish. Development 107: 201-213.
Sander, K. 1976. Specification of the basic body pattern in insect embryogenesis. Adv. Insect Physiol. 12: 125-238.
Sokoloff, A. 1972. "The Biology of Tribolium." vol. 1. Oxford Press, London. pp 300.
Struhl, G. 1981. A homeotic mutation transforming leg to antenna in *Drosophila*. Nature 292: 635-638.
Tazima, Y. 1964. "The Genetics of the Silkworm." Academic Press, London.

MOLECULAR BIOLOGY OF CHOLINERGIC SYNAPSES IN INSECTS

H. Breer

University of Stuttgart-Hohenheim
Institute of Zoophysiology
7000 Stuttgart 70, West Germany

INTRODUCTION

Functions of the nervous system are based on the specific communication between neurons which takes place at specialized cellular contacts, the synapses. In insect head and thoracic ganglia synaptic contacts are restricted to the central neuropil region, where axons and dendrites, derived from the intrinsic motor- and interneurons, as well as from the arborizing axons of the peripheral sensory neurons, form a mass of interwoven processes with numerous synaptic contacts. The cell bodies of intrinsic neurons, located outside the neuropil, appear to be free of synapses. Synaptic interaction between nerve cells is mediated by neurotransmitters. Comparing the concentration of some classical neurotransmitters in the nervous system of insects reveals that acetylcholine (ACh) is by far the predominant transmitter (Table 1), and ACh concentrations are much higher than in the vertebrate brain (Pitman, 1971). The preponderance of cholinergic synapses in insect ganglia may be due to the fact that in different phyletic lines, cholinergic synapses are localized in different parts of the nervous system. In the peripheral nervous system of vertebrates, only efferent, centrifugal nerves are cholinergic, whereas the sensory neurons are noncholinergic. In arthropods, in contrast, sensory and some central neurons are cholinergic and the efferent neurons are not (Florey and Michelson, 1973).

Detailed knowledge of the metabolism and functional role of acetylcholine, as well as extensive studies exploring the molecular processes and elements of cholinergic transmission, may lead towards an understanding of molecular functions of the insect nervous system and may elucidate interesting aspects of insect neuropharmacology.

CHOLINERGIC SYNAPSES

At cholinergic synapses, synthesis of acetylcholine from choline and acetyl-CoA is catalyzed by choline acetyltransferase (ChAT). The neurotransmitter is translocated into synaptic vesicles and is released from cholinergic nerve terminals upon stimulation in a Ca^{2+}-dependent process. The released chemical messenger transiently interacts with specific receptors, causing changes in membrane permeability. ACh is subsequently inactivated by the hydrolytic enzyme acetylcholinesterase (AChE). The resulting choline is preferentially taken up into the presynaptic terminal via specific high-affinity carrier mechanisms, and most of it is immediately converted back to ACh. A typical cholinergic synapse is characterized chemically by an ACh concentration of 0.3-3 mM, by the presence of ChAT, and by a specific choline carrier. The first report of ACh in insect nervous tissue was made by Gautrelet (1938), who demonstrated the presence of choline ester in several insect species. Shortly after the discovery of ACh, the enzymatic activities for synthesis (Tobias et al., 1946), as well as for hydrolyzing (Mikalonis and Brown, 1941) the choline ester, were established (Table 1).

Molecular Insect Science, Edited by
H. H. Hagedorn *et al.*, Plenum Press, New York, 1990

Table 1. Comparison of cholinergic activities in the nervous tissue of mouse and locust

CHOLINERGIC ACTIVITIES	MOUSE CEREBELLUM	MOUSE CORTEX	LOCUST HEAD GANGLIA
Acetylcholine (nmol/mg)	0.17±0.05	0.3±0.1	6.8±1.0
Choline acetyltransferase (pmol/mg/min)	13.3±1.5	62.3±4.5	520±18
Acetylcholinesterase (nmol/mg/min)	24.3±2.3	104.2±7.8	784±25
Choline uptake (pmol/mg/min)	0.35±0.05	1.4±0.2	116±10
Hemicholinium binding (fmol/mg)	4.1±0.5	25.0±3.1	1200±150

Data are the means of 3-5 determinations ± SD

ENZYMES OF THE CHOLINERGIC SYSTEM

Choline acetyltransferase

In cholinergic nerve cells, acetyl-CoA provides acetyl groups and energy for the enzymatic acetylation of choline. Choline acetyltransferase is a key element and a marker of cholinergic neurons. Comparative studies have shown that ChAT activity is more than 100 times higher in insect ganglia than in vertebrate brain (Breer, 1981a). As in other animals, most of the enzyme activity in insects is found in the soluble fraction, suggesting that ChAT is a cytoplasmic enzyme (Fonnum, 1967), although there is experimental evidence that a considerable portion of ChAT is bound to membranes. It has in fact been suggested that a functional complex, composed of choline carrier and membrane-bound ChAT, may account for a rapid acetylation of choline. Choline acetyl-transferase from *Drosophila melanogaster,* a monomeric globular protein with a molecular weight of about 67 kDa, has been purified to homogeneity (Slemmon et al., 1982). Subsequently, ChAT-cDNA has been cloned and sequenced (Itoh et al., 1986). The enzyme's mRNA is estimated to be approximately 4.7 kb long. The cloned cDNA hybridized only to the region 91B-D on polytene chromosome 3R, thus confirming the localization of the single gene (Cha) for ChAT identified by aneuploidy (Hall and Greenspan, 1979).

Acetylcholinesterase

At cholinergic synapses it is important that released neurotransmitter be rapidly removed from the synaptic cleft and from the site of action on the postsynaptic membrane. This essential prerequisite is realized by acetylcholinesterase, which hydrolyzes ACh to physiologically inactive products. AChE is very active in insect nervous tissue and is found in multiple forms. The AChE polymorphism is obviously due to aggregation of active forms which were identified as 80 kDa and 110 kDa polypeptides (Silver and Prescott, 1982). The gene coding for AChE (Ace) was identified in *D. melanogaster* (Kankel and Hall, 1976) and localized in a small segment of the right arm of chromosome 3. Thus, the genes for the two cholinergic enzymes are both located in the same region.

ISOLATION OF NERVE TERMINALS

A major advance in studying dynamic processes of synapses and synaptic transmission was made by the discovery that nerve terminals were excised by homogenizing nervous tissue under conditions of moderate shear in isotonic solutions. Modifications of the original procedure, which cope with the fragility of insect nerve terminals, made it possible to isolate morphologically intact, viable nerve endings (synaptosomes) from insect ganglia (Breer,

1981b). Comparisons of the distribution of cholinergic elements in subcelluar fractions, showed that all components of the cholinergic system were highly enriched in the synaptosomal fraction, indicating that insect synaptosomal preparations contain high proportions of cholinergic nerve endings. Thus synaptosomes from insects appear to be suitable for exploring molecular aspects of cholinergic synapses.

CHOLINE UPTAKE

The availability of exogenous choline is an essential prerequisite for the function of cholinergic synapses. Choline is accumulated via a specific, high-affinity carrier in the presynaptic membrane and converted to ACh (Tucek, 1985). The high-affinity uptake of choline appears to be unique to cholinergic nerve endings, sequestering the choline derived from hydrolysis of ACh released into the synaptic cleft. Uptake of choline via this pathway seems to be the rate-limiting, regulatory step for ACh synthesis (Kuhar and Murrin, 1978), and is thus of particular importance in cholinergic synaptic function. Pharmacological studies have indicated that the transport of choline is efficiently blocked by hemicholinium-3 (HC-3), which is now considered as the inhibitor of the high-affinity choline carrier. Radiolabeled hemicholinium can be used as specific ligand for the choline carrier; HC-3 binding is saturable, reversible, sodium-dependent, and blocked by choline. The high number of binding sites found in insect preparations is in line with the high activities of other molecular components in cholinergic synapses (Knipper et al., 1989a).

The molecular mechanism of choline translocation mediated by a high-affinity carrier is still poorly understood, partly owing to the problem of experimentally separating this function from metabolic activities and the complex compartmentation of intact nerve endings. Synaptosomal ghosts, obtained by spontaneous revesiculation of isolated presynaptic membranes from insect nervous tissue, unlike preparations from mammalian brain (Meyer and Cooper, 1982), can accumulate choline, notably via high-affinity pathways, with artificially imposed ion gradients as the sole energy source, when loaded with K^+-ions and resuspended in NaCl (Breer, 1983). Manipulation of the intra- and extravesicular ion composition has established that Na^+ and Cl^- gradients are the main driving force (Breer and Lueken, 1983).

Because of its key role in the function of cholinergic synapses, high-affinity choline uptake is supposed to be regulated by various mechanisms. There are some indications that the uptake rate is modified by synaptic activity (Barker, 1976), but recent experiments suggest a much more subtle form of regulation. Modification of the intrasynaptosomal level of cyclic nucleotide, either by activating adenylate cyclase via presynaptic receptors or by incubating with permeable cAMP-derivatives, significantly increases the rate of choline accumulation by insect synaptosomes. The effects of deoxy-cAMP-derivatives suggest that an activation of kinases by cAMP may be involved in the reaction cascade (Breer and Knipper, 1990). The stimulatory actions of phorbol esters, known to activate protein kinase C, suggests that other phosphorylation pathways may also affect the transport system for choline (Knipper and Breer, 1987). The obvious question is whether changes in choline transport activity induced by kinase reactions result from alteration of the intrinsic carrier activity, or are due instead to changes in the actual number of functional carriers in the plasma membrane. This problem was approached in binding studies using tritiated HC-3 as a specific ligand. Pretreatment of synaptosomal preparations with either permeable cAMP-derivatives or phorbol esters significantly increased the number of HC-3 binding sites in synaptosomal membranes. As carrier proteins cannot be synthesized in isolated nerve terminals, the apparent increase of choline carrier suggests that preexisting, occult carrier proteins may be exposed or inserted into the plasma membrane upon phosphorylation. Whether this includes a phosphorylation of the carrier itself can be evaluated only when the molecular constituents of the transporter have been identified.

Using photoaffinity tritiated HC-3 could be cross-linked to its specific binding site, which was then identified as a single polypeptide with a molecular mass of about 85 kDa (Knipper et al., 1989a). In an alternative approach, monoclonal antibodies have been generated that specifically block the high-affinity, Na^+-dependent transport of choline in insect synaptosomes and membrane vesicles. These antibodies label a similar 85 kDa polypeptide on Western blots, and they also stain distinct areas in the sensory neuropil of insect ganglia as shown in Fig. 1 (Knipper et al., 1989b). In recent experiments, the antigen has been purified to homogeneity by immunoaffinity-chromatography and subsequently

reconstituted in liposomes. Only proteoliposomes containing the putative carrier-protein accumulated choline via a high-affinity, sodium-dependent process that was blocked by HC-3 (Knipper et al., 1989c), thus demonstrating that the identified polypeptide represents a constituent of the choline transporter.

RELEASE OF ACETYLCHOLINE

The release of neurotransmitters from presynaptic terminals - a fundamental step in synaptic transmission - is normally triggered by the arrival of a nerve impulse. The complex organization of insect ganglia often makes it impractical to use *in situ* preparations for studying the mechanisms of ACh release. Synaptosomes from insects, which are able to accumulate choline and convert it to ACh, have provided a useful preparation for studying aspects of transmitter release from central cholinergic synapses. The release can be monitored by means of a microperfusion technique (Breer and Knipper, 1985), which prevents reuptake and allows continuous measurement of released material. Whereas perfusion with isotonic medium causes only very little efflux, the addition of depolarizing agents induces a considerable Ca^{2+}-dependent release of ACh (Breer and Knipper, 1984).

In the course of perfusion experiments, it became evident that the presence of elevated levels of ACh in the perfusion medium, significantly diminished the rate of release, suggesting that ACh can influence its own release, possibly via feedback mechanisms. The application of various cholinergic agents has demonstrated that muscarinic agonists, like oxotremorine, reduce the output of ACh, whereas muscarinic antagonists enhance evoked release. These results appear to support the hypothesis advanced by Polak (1971), that feedback regulation of ACh-release is exerted via muscarinic autoreceptors located on cholinergic terminals. Subsequent experiments indicated that muscarinic agonists significantly reduce the activity of adenylate cyclase and lower the concentration of cAMP in insect synaptosomes (Knipper and Breer, 1988; 1989). The hypothesis that autoregulation of ACh-release is mediated by changes in the intrasynaptic cAMP-level is supported by the observation that permeable analogues of cAMP, such as 8-Br-cAMP, antagonize the attenuation effects of muscarinic agonists (Knipper and Breer, 1989). Permeable cAMP analogues enhance the release rate in a dose-dependent manner, whereas the closely related analogue 2-deoxy-cAMP, which binds, but does not activate the regulatory subunit of protein kinases, reduces the rate of ACh-release, indicating that protein phosphorylation may be involved in modulation of the release process. This view is supported by the observation that activation of protein kinase C via phorbol esters also enhances the rate of ACh-release. Efforts to identify the intrasynaptic kinase substrates that mediate the modulatory effects, and may play a central role in the stimulus-secretion process of transmitter release, are of considerable interest with regard to understanding the regulation of synaptic activities.

RECEPTORS FOR ACETYLCHOLINE

Receptors for ACh are important at cholinergic synapses for recognition and transduction of the chemical signal, released ACh. The existence of specific receptors for ACh in insects was first evident in the high sensitivity of certain insect neurons to applied ACh following the inhibition of endogenous AChE (Kerkut et al., 1969; Sattelle, 1980). Two different types of receptors can be distinguished, which differ in mode of action as well as in pharmacology: nicotinic and muscarinic receptors. Both receptor types can be analyzed and identified by means of specific ligands: α-bungarotoxin (BGTX) is used as a ligand for the nicotinic receptor, and quinuclidinic benzylate (QNB) for muscarinic receptors. Comparative binding studies revealed that the concentration of putative receptors for acetylcholine (AChR) is much higher in insects than in vertebrates, which parallels the distribution of other cholinergic elements. It is most remarkable, however, that in each case a different receptor type predominates: in mouse brain, mostly QNB-binding sites (muscarinicAChR) are found, while insect ganglia contain very high concentrations of α-toxin binding sites (putative nicotinic AChR). The functional implications of this contrasting deployment of of AChR levels in insects and vertebrates have yet to be evaluated (Breer and Sattelle, 1987).

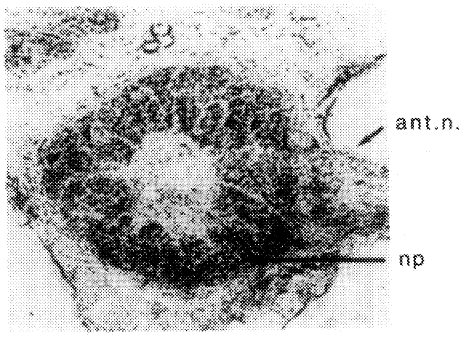


Fig. 1 Immunocytochemical localization of high affinity choline carrier in antennal lobe. np, neuropil; ant. n. antennal nerve.

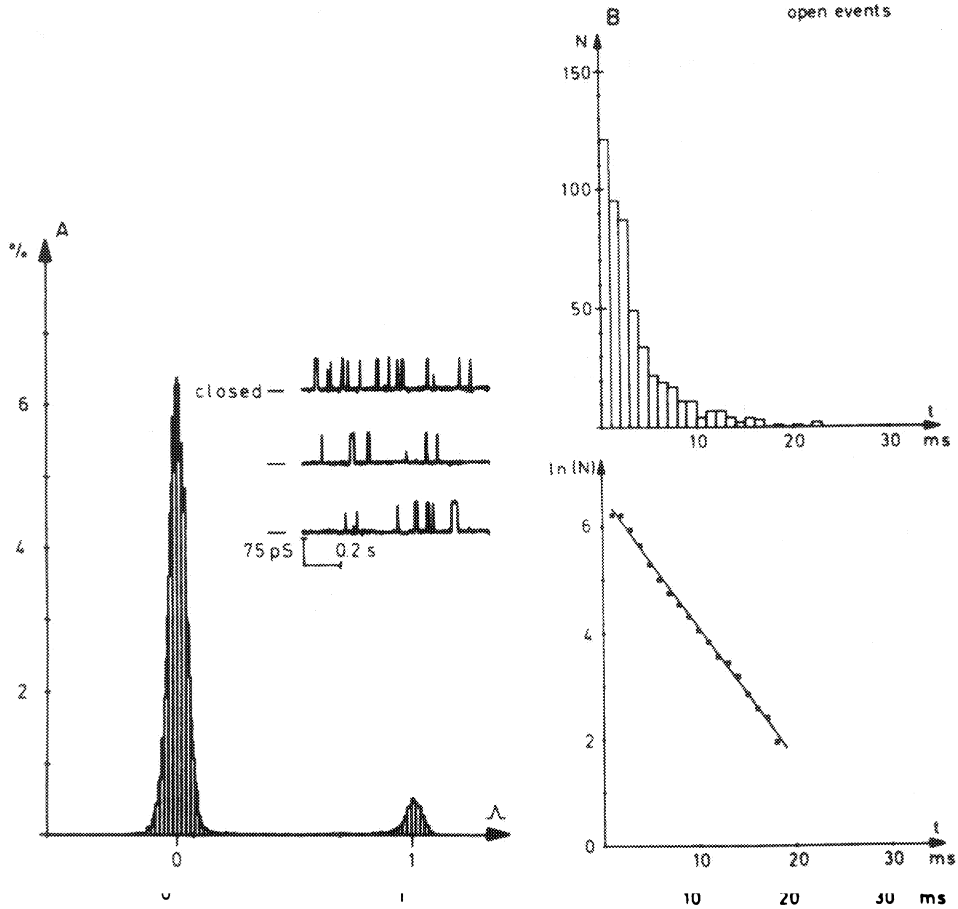


Fig. 2 Evaluation of the single-channel properties of the purified neuronal actylcholine receptor reconstituted in planar lipid bilayer. The amplitude histogram demonstrates well defined closed (0) and open states (1). The lifetime distribution of the open state is shown in a linear (top) and logarithmic plot; the lifetime is single exponentially distributed, giving a mean open time of about 4 msec.

Muscarinic Receptors

In binding studies using a variety of preparations and ligands, the pharmacological profile of insect muscarinic receptors has been established although the potency of muscarinic drugs seems somewhat lower than that in vertebrates. Alkylating muscarinic antagonists, such as [^{3}H]propylbenzylcholine mustard, were used for affinity labeling of muscarinic receptors from fly heads and locust ganglia. It each case a single polypeptide (Mr 75-80 kDa) was specifically labeled (Venter et al.,1984; Knipper and Breer, 1988). The receptor size was confirmed in radiation-inactivation experiments on cockroach muscarinicAChR (Lummis et al., 1984). In flies this polypeptide strongly cross-reacted with monoclonal antibodies against rat-brain muscarinic receptors, indicating structural relatedness of these receptors. This notion was emphasized when the primary structure of the muscarinic receptor from Drosophila was recently shown (Onai et al., 1989) to exhibit a high degree of structural homology to mammalian muscarinic receptors. The receptor gene was mapped to a single site at the tip of the right arm of chromosome 2 in the region 60C5-8 and is thus located on a chromosome different from that carrying the genes for the cholinergic enzymes.

Based on different affinities for the characteristic ligand pirenzipine, there are at least two major subtypes of muscarinicAChR, which are coupled to different second messenger systems. The M1-type, with high affinity for pirenzipine, is coupled to the phosphoinositol-system, whereas the M2-type, with low affinity for pirenzipine, induces down-regulation of adenylate cyclase. It has been established in binding studies that both subtypes are present in insect nervous tissue (Knipper and Breer, 1988). In binding studies using subcellular fractions, as well as in recent electrophysiological approaches, it has been revealed that the M1 receptors are preferentially located in nerve cell somata, whereas the nerve terminals contain mainly M2 receptors (Knipper and Breer, 1988; Benson, 1989). These data are in line with the notion that the modulatory effect of muscarinic agonists on presynaptic activities (see above; Hue et al., 1989) probably is mediated via M2 receptors and involves attenuation of the activity of adenylate cyclase and the level of cAMP.

Nicotinic Receptors

Electrophysiological studies have revealed that nicotinic receptors are distributed on the synapse-free cell bodies of neurons as well as throughout the synapse-rich neuropil. Moreover, cholinergic synaptic transmission is irreversibly blocked by nanomolar concentrations of α-bungarotoxin (Sattelle et al., 1983). Thus, in insects, the toxin- binding sites are associated with functional nicotinic AChR. This view was supported in precipitation experiments demonstrating that solubilized ACh-binding sites are completely precipitated by Sepharose-coupled α-toxins in insects and Torpedo (but only partially in mouse) brain preparations (Benke and Breer, 1989a).

In experiments using BGTX as a high-affinity probe, the binding constants obtained closely resembled those reported for other species (Breer and Sattelle, 1987, and the pharmacological profile showed that the binding site is most strongly inhibited by nicotinic drugs. The relative potencies of different cholinergic ligands were quite similar to those reported for vertebrate neuronal binding sites but showed remarkable differences compared to results obtained with the Torpedo receptor (Benke and Breer, 1989a).

Endogenous Modulators of Acetylcholine Receptors

Owing to the high-affinity binding of α -BGTX to neuronal AChRs, it has been speculated that endogenous ligands may exist for these binding sites. From locust nervous tissue low molecular weight compounds have been purified that competitively inhibit the binding of α-toxins as well as ACh to neuronal membranes from insects and vertebrates (but not from electric tissue) in a dose-dependent manner. These heat-stable compounds were identified as positively charged amines that may function as modulators for insect neuronal AChRs (Benke and Breer, 1989b).

During the course of binding studies it was observed that removal of polypeptides associated with membrane reversibly increased the number of BGTX-binding sites and decreased the number of binding sites for ACh in neuronal membrane preparations, suggesting the existence of endogenous membrane-associated factors that might allosteric-

ally modulate the ligand-binding sites of the receptor protein. The regulatory factor was purified and identified as a 20 kDa polypeptide associated with membrane that increased the number of accessible binding sites for ACh when added to neuronal membrane preparations. The purified polypeptide was found to be phosphorylated by cAMP-dependent protein kinase and to lose its modulatory function upon phosphorylation. Thus, it is conceivable that the number of functional AChRs in neuronal membranes may be controlled and fine-tuned via regulatory polypeptides.

Molecular Properties

A number of alternative and complimentary procedures have been employed to elucidate the molecular properties of the insect nicotinic AChR. All approaches confirmed that the receptor apparently represents a macromolecular complex ($M_r \approx 300$ kDa) composed of several subunits. Despite some controversy concerning the number and sizes of the subunits, there is some evidence that the insect receptor may represent a homo-oligomeric complex (Breer et al., 1985). Based on immunological cross-reactivity and some N-terminal sequence, a considerable degree of structural homology to vertebrate receptor subunits has been proposed. With the aid of monospecific antisera, the purified receptor from locust was topochemically localized in the central neuropil, indicating that this receptor may be of synaptic origin.

Reconstitution

The reconstitution of purified receptor proteins in artificial lipid membranes is considered to be the appropriate approach ultimately to demonstrate that the isolated polypeptides represent functional receptors, i.e. ligand-regulated cation-selective channels. ACh-receptor protein, isolated by affinity chromatography from locust nervous tissue, has been reconstituted into planar lipid bilayers (Hanke and Breer, 1986). Upon addition of cholinergic agonists, the fluctuation of ion channels with a conductance of about 75pS and an open time of a few msec was observed (Fig. 2), demonstrating that the purified protein represents a functional nicotinic AChR. In a more detailed analysis, characteristic features of this neuronal AChR-channel emerged (Hanke and Breer, 1987). The cationic channel displayed a mean open time of about 4 msec, which depended only slightly on the agonist concentration, whereas the closed-state lifetime decreased significantly with increasing agonist concentration. Open- and closed-state lifetimes were single exponentially distributed at low agonist concentration. Multiple-gating events, bursting behavior, and eventually desensitization were observed at increasing concentrations. Pharmacological experiments revealed that neither d-tubocurarine nor hexamethonium affects the conductance of the channel. Instead, both assays reduce its probability of opening. The effectiveness of hexamethonium on channel activity was found to be quite low, confirming results from ligand-binding and electrophysiological studies and indicating the pharmacological integrity of the purified receptor protein.

CONCLUSION

The application of refined and miniaturized advanced techniques in exploring molecular aspects of cholinergic synaptic transmission and functional elements of cholinergic nerve contacts in the insect nervous system has contributed to a more detailed understanding of cholinergic signal transmission between nerve cells in general. These results emphasize the usefulness of insects and invertebrates for studies of general problems of cellular and molecular neurobiology. Experiments using insect material have provided opportunities to use synaptosomal ghosts and monoclonal antibodies for studying functional and molecular properties of the choline transporter; furthermore, an affinity-purified α-toxin-binding protein isolated from insect nervous tissue was the first neuronal nicotinic acetylcholine receptor to be functionally reconstituted in a planar lipid bilayers. Preparations from cholinergic synapses of insect ganglia (organelles, membranes, molecules) may be suitable for developing and standardizing simple assay systems for screening the targets and for analyzing the mode of action of putative insecticides. Thus, detailed knowledge of cholinergic synaptic elements and processes may contribute to the design and development of more selective and safer pest control agents in the future.

REFERENCES

Barker, L. A. 1976. Modulation of synaptosomal high affinity choline transport. Life Sci. 18: 725-732.

Benke, D., and Breer, H.. 1989a. Comparison of acetylcholine and α-bungarotoxin binding sites in insects and vertebrates. Comp. Biochem. Physiol. 94C: 71-80.

Benke, D., and Breer, H. 1989b. Endogenous low molecular weight inhibitors of cholinergic binding sites. Insect Biochem. 19: 423-430.

Benson, J. A.. 1989. Ml-like muscarinic receptors mediate cholinergic activation of an inward current in isolated neuronal somata from locust thoracic ganglia. Soc. Neurosci. Abs. 15: 365.

Breer, H. 1981a Comparative studies on cholinergic activities in the central nervous system of *Locusta migratoria*. J. Comp. Physiol. 141: 271-275.

Breer, H. 1981b. Characterization of synaptosomes from the central nervous system of insects, Neurochem. Int. 3: 155-163.

Breer, H. 1983. Choline transport by synaptosomal membrane vesicles isolated from insect nervous tissue. FEBS Lett. 153: 345-438.

Breer, H., and Knipper, M. 1984. Characterization of acetylcholine release from insect synaptosomes, Insect Biochem. 14: 337-344.

Breer, H., and Knipper, M. 1985. Synaptosomes and neuronal membranes from insects, in: "Neurochemical Techniques in Insect Research," Breer, H., Miller, T. A. eds., Springer, New York, pp 125-154.

Breer, H., Kleene, R. and Hinz, G. 1985. Molecular forms and subunit structure of the acetylcholine receptor in the central nervous system of insects. J. Neurosci. 5: 3385-3392.

Breer, H., and Knipper, M. 1990. Regulation of high affinity choline uptake. J. Neurobiol. 21: 269-275.

Breer, H., and Lueken, W. 1983. Transport of choline by membrane vesicles prepared from synaptosomes of insect nervous tissue. Neurochem. Int. 5: 713-720.

Breer, H., and Sattelle, D. B. 1987. Molecular properties and functions of insect acetylcholine receptors. J. Insect. Physiol. 33: 771-790.

Florey, E., and Michelson, M. J. 1973. Occurrence, pharmacology and significance of cholinergic mechanisms in the animal kingdom, pp. 11-41, in: "Comparative Pharmacology," M. J. Michelson, ed., Pergamon Press, Oxford.

Fonnum, F. 1967. Choline acetyltransferase: binding to and release from membranes. Biochem. J. 109: 389-398.

Gautrelet, J. 1938. Existence d'un complex d'acetylcholine dans le cerveau et divers organes. Ses caracteres, sa repartition. Bul. Acad. Nat. Med. 120: 285-291.

Hall, J. F., and Greenspan, R. J. 1979. Genetic analysis of *Drosophila* neurobiology. Annu. Rev. Genet. 13: 127-195.

Hanke, W., and Breer, H. 1986. Channel properties of an insect neuronal acetylcholine receptor protein reconstituted in planar lipid bilayers. Nature, Lond. 321: 171-174.

Hanke, W., and Breer, H.. 1987. Characterization of the channel properties of a neuronal acetylcholine receptor reconstituted into planar lipid bilayers. J. Gen. Physiol. 90: 855-879.

Hue, B., Lapied, B. and Malecot, C.O. 1989. Do presynaptic muscarinic receptors regulate ACh release in the CNS of the cockroach, *Periplateta america*? J. Exp. Biol. 142: 447-451.

Itoh, N., Slemmon, J. R., Hawke, D. H., Williamson, R., Morita,E., Itakura, K., Roberts, E., Shively, J. E., Crawford, G.D., and Salvaterra, P. M. 1986. Cloning of Drosophila choline acetyltransferase cDNA. Proc. Natl. Acad. Sci. (USA) 83: 4081-4085.

Kankel, D. R., and Hall, J. F. 1976. Fate mapping of nervous system and other internal tissue in genetic mosaic of *Drosophila melanogaster*. Dev. Biol. 48: 1-24.

Kerkut, G. A., Pitman, R. M., and Walker, R.J. 1969. Iontophorectic application of acetylcholine and GABA onto insect central neurons. Comp. Biochem. Physiol. 31: 611-633.

Knipper, M., and Breer, H. 1987. Protein kinase C in the nervous system of insects: effects of phorbol esters on cholinergic synapses. Neurochem. Int. 10: 323-328.

Knipper, M., and Breer, H. 1988. Subtypes of muscarinic receptors in insect nervous system. Comp. Biochem. Physiol. 90C: 275-280.

Knipper, M., and Breer, H. 1989. Muscarinic receptors modulating acetylcholine release from insect synaptosomes. Comp.Biochem. Physiol. 93C: 287-292.

Knipper, M., Krieger, J., and Breer, H. 1989a. Hemicholinium-3 binding sites in the nervous system of insects. Neurochem.Int. 14: 211-215.

Knipper, M., Strotmann, J., Mdler, U., Kahle, C., and Breer, H. 1989b. Monoclonal antibodies against the high affinity choline transport system. Neurochem. Int. 14: 217-222.

Knipper, M., Boekhoff, I., and Breer, H. 1989c. Isolation and reconstitution of the high-affinity choline carrier. FEBS Lett. 245: 235-237.

Kuhar, M. J., and Murrin, L. C. 1978. Sodium-dependent high affinity choline uptake. J.Neurochem. 30: 15-21.

Lummis, S. C. R., Sattelle, D. B., and Ellory, J. C. 1984. Molecular weight estimates of insect cholinergic receptors by radiation inactivation. Neurosci. Lett. 44: 7-12.

Meyer, E. M., and Cooper, J. R. 1982. High-affinity choline transport in proteoliposomes derived from rat cortical synaptosomes. J. Neuroscience 3: 987-994.

Mikalonis, S. J. and Brown, R. H. 1941. Acetylcholine and acetylcholinesterase in the insect central nervous system. J. Cell. Comp. Physiol. 18: 401-403.

Onai, T., Fitzgerald, M.G., Arakawa, S., Gocayne J.D., Urquhart,D.A., Hall, L.M., Fraser, C.M., McCombie, W.R., and Venter, J.C. 1989. Cloning, sequence analysis and chromosome localization of a *Drosophil*a muscarinic acetylcholine receptor. FEBS Lett. 255: 219-224.

Pitman, R. M. 1971. Transmitter substances in insects: A review. Comp. Gen. Pharmacol. 2: 347-371.

Polak, R. L. 1971. The stimulating effect of atropine on the release of acetylcholine by rat cerebral cortex *in vitro*. Brit. J. Pharmacol. 14: 600-606.

Sattelle, D. B. 1980. Acetylcholine receptors of insects. Adv. Insect Physiol. 15: 215-315.

Sattelle, D. B., Harrow, I. D., Hue, B., Pelhate, M., Gepner, J.I., and Hall, I. M. 1983. α-bungarotoxin blocks excitatorysynaptic transmission between cercal sensory neurons and giant interneurone 2 of the cockroach *Periplaneta americana*. J. Exp. Biol. 107: 473-489.

Silver, L. H., and Prescott, D. J. 1982. Aggregation propertiesof the acetylcholin-esterase from the central nervous systemof *Manduca sexta*. J. Neurochem. 38: 1709-1717.

Slemmon, J. R., Salvaterra, P. M. Crawford, G.D., and Roberts, E. 1982. Purification of choline acetyltransferase from *Drosophila melanogaster*. J. Biol. Chem. 257: 3847-3852.

Tobias, J. M., Kollross, H. H. and Savit, J. 1946. Acetylcholine and related substances in the cockroach, fly and crayfish, and the effect of DDT. J. Cell. Comp. Physiol. 28: 159-182.

Tucek, S. 1985. Regulation of acetylcholine synthesis in the brain. J. Neurochem. 44: 11-18.

Venter, C. J., Eddy, B., Hall, L. M. and Fraser, C. M. 1984. Monoclonal antibodies detect the conservation of muscarinic cholinergic receptor structure from *Drosophila* to human brain and detect possible structural homology with α-adrenergic receptors. Proc. Natl. Acad. Sci. USA 81: 272-276.

ISOLATION, CHARACTERIZATION AND PROGRESS IN CLONING OF CYCLODIENE INSECTICIDE RESISTANCE IN *Drosophila melanogaster*

R.H. ffrench-Constant[1], R.T. Roush[1] and R.J. MacIntyre[2]

[1]Department of Entomology, Comstock Hall and
[2]Section of Genetics and Development, Biotechnology Building
Cornell University, Ithaca, N.Y. 14853

INTRODUCTION

Insecticide resistance is an increasingly serious problem for the control of medically and agriculturally important pests. More than 447 species of arthropods have evolved resistance to one or more pesticides; some 17 species have shown resistance to all five major classes of insecticides often outstripping the rate at which new pesticides (or even non-pesticidal controls) can be introduced to replace them (Georghiou 1986). Insecticide resistance is also a dramatic example of adaptation at the molecular level. The majority of cases of practically significant resistance in the field are due to single major genes (Roush & McKenzie 1987, Roush and Daly 1990), that either increase the metabolism of pesticides or cause insensitivity in the target site (Soderlund & Bloomquist 1990).

Although there have been several successful studies on the molecular genetics of metabolic resistances such as elevated esterases (Field et al. 1988) and the mixed function oxidase system (Feyereisen et al. 1989); and the altered target site mechanism, insensitive acetylcholinesterase (Berge and Fournier 1988), there has been little progress in studying mechanisms where gene products are unknown or difficult to isolate, as in nerve insensitivity to pyrethroids (and DDT) and to cyclodiene insecticides. The ideal species in which to study such genes would be one where the cytological location of such genes could be identified thereby facilitating their cloning, and where a transformation system is available for confirmation of cloning.

For these and other reasons, Wilson (1988) suggested that *Drosophila melanogaster* is the ideal model species for the study of insecticide resistance. Unfortunately, there has been little progress in the cloning of resistance genes in this insect, primarily because it has been difficult to identify genes that are homologous to those in other insects. With the exception of resistance conferred by mixed function oxidases (Waters and Nix 1988, Houpt et al. 1988) and methoprene resistance (Wilson and Fabian 1986), resistances described in *D. melanogaster* bear little similarity to the high levels of resistance found in other insects. For example, resistance in *D. melanogaster* to carbamates and organophosphorus insecticides caused by an insensitive acetylcholinesterase (Morton and Singh 1982, Berge and Fournier 1988) or to pyrethroids via altered numbers of sodium channels (Kasbekar and Hall 1988) are low level.

In particular, there have been no reports of high levels of cyclodiene resistance in *D. melanogaster* which is surprising since cyclodiene resistance accounts for over 60% of cases of resistance in insects and mites (Georghiou 1986). In addition, the presence of such resistance in other diptera such as the house fly *Musca domestica,* and Australian sheep blowfly, *Lucilia cuprina,* is well documented (Foster et al., 1981). In fact, because cyclodiene resistance is found in more species than any other kind of resistance (Georghiou 1986) and appears to be similar in all species studied (Oppenoorth 1985, Yarbrough et al., 1986), it may be the best example of molecular evolution ever driven by man.

Molecular Insect Science, Edited by
H. H. Hagedorn *et al.*, Plenum Press, New York, 1990

Although the $GABA_A$ receptor is the proposed primary target for cyclodiene insecticides (Eldefrawi and Eldefrawi 1987), the precise mode of action of cyclodienes and the mechanisms by which insects become resistant to them remain obscure. The GABA receptor remains an important molecular target for novel insecticides and thus isolation and cloning of cyclodiene resistance mutants may aid in further characterizing this target site. Despite the cloning of the vertebrate $GABA_A$ receptor (Schofield et al. 1987), little progress has been made on cloning GABA genes from insects.

Here we describe the isolation of a strain with high levels of cyclodiene resistance from field populations of *D. melanogaster* and examine the pattern of resistance to various cyclodienes in relation to those found in other resistant insects. Chromosomal localization, deficiency mapping and progress in a chromosomal walk to clone the gene responsible for resistance are discussed.

MATERIALS AND METHODS

Field collection and screening

Several thousand *D. melanogaster* were collected from decaying tomatoes, peaches, melons and apples in agricultural areas of Maryland and New York. Preliminary screening for resistance was carried out in 450 ml glass jars, the insides of which were coated with 30-100 µg of dieldrin in acetone. After a 24 hr exposure, survivors were transferred to a standard cornmeal diet. Their progeny were further selected as males and virgin females until they could survive a dose of 300 µg of dieldrin applied to the insides of 20 ml glass scintillation vials. This was repeated for four generations at which stage the resistant strain was assumed to be homozygous for a gene or genes conferring resistance.

Chromosomal localization

Crosses and bioassays of offspring were conducted to examine the dominance of resistance, the number of genes involved, and the extent of resistance to other cyclodienes and related compounds. Approximately 20-30 flies were tested in each scintillation vial. Mortality was assessed after 24 hrs. To establish which chromosome/s carried the gene/s responsible for resistance, crosses involving stocks carrying pairs of markers for the second and third chromosomes [Bristle (Bl), Lobe (L); and Lyra (Ly), Stubble (Sb) respectively] were used (unpublished).

After recombinational mapping, seven overlapping deficiency stocks (kindly provided by the Mid America *Drosophila* Stock Centre at Indiana University) were examined in order to further localize the gene. Homozygous resistant (*R/R*) flies were crossed to each deficiency (over a TM3 balancer, *Df(3L) /TM3*) in turn, and their *R /Df* and *R /TM3* progeny screened at 30 µg dieldrin (ffrench-Constant and Roush, unpublished).

Characterization of cross-resistance

Following localization of the resistance gene, the effects of mixed function oxidase genes also present in the field strains were removed by backcrossing to a standard susceptible strain, Oregon R (here denoted as *S/S*. Dominant markers were used to ensure sequential replacement of chromosomes from the resistant strain with those from Oregon R (ffrench-Constant and Roush, in press). The resistance of this strain (MD-BC5) was compared to that of Oregon R for propoxur, malathion and DDT (to which mixed function oxidases confer resistance in *D. melanogaster* as shown by our unpublished data) and several GABA antagonists.

Cosmid walk

A cosmid genomic library made from flies isogenic for all three chromosomes (strain iso-1) was obtained from John Tamkun, University of Colorado. The cosmid vector is a modified form of CosPeR, with *Not*l sites flanking the *Bam*Hl cloning site. As *Not*l is a relatively rare restriction site in *D. melanogaster* and certain sites such as *Eco*Rl, *Xba*l and *Hpa*l are absent from the vector, mapping and isolation of end fragments using these enzymes is simplified.

Approximately 25,000 transformants were plated out onto nitrocellulose filters on ampicillin plates (40 μg/ml) at a density of 3000 bacteria/150 mm plate. Replica filters were made and the master filters stored at -20^0 C on ampicillin plates (40 μg/ml) containing 15% glycerol. Bacteria on the replica filters were lysed by leaving filters on chloramphenicol plates (250 μg/ml) for 20 hours. DNA was then fixed to the filters by alternately soaking filters in the following solutions: a) 0.5M NaOH, 1.5M NaCl, b) lM Tris HCl (pH 7.5) and c) 0.5M Tris HCl (pH 7.4), 1.5M NaCl. Filters were hybridized with primer extended phage or cosmid probes in standard hybridization mixes at 65 ^{0}C, washed repeatedly at 65 ^{0}C and exposed to X-ray film (for details of hybridization and washing see Dillela and Woo 1985). Master filters were repeatedly used for isolation of putative positive cosmids, which enabled re-identification of previously extracted positive cosmids. Re-screens of candidate positives were conducted by scratching the area surrounding the positive colony with a wooden toothpick and then transferring the bacteria to Luria Broth containing 40 μg/ml ampicillin, growing at 37 ^{0}C for 30 min and re-plating, hybridizing and washing as above.

A chromosomal walk was carried out by repeatedly identifying end fragments from the cosmid inserts and re-screening the library to identify overlapping cosmids with the least homology as shown by Southern blotting. Progress of the walk, and initial directionality, was monitored by *in situ* hybridization to wild-type Canton S polytene chromosomes (Pardue, 1986).

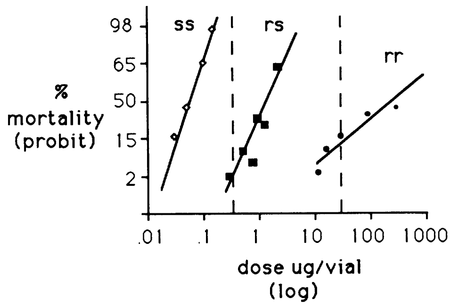


Fig. 1. Dose response lines with dieldrin for homozygous susceptible (*S/S*), heterozygous (*R/S*) and homozygous (*R/R*) cyclodiene resistant (BC5) flies.

RESULTS AND DISCUSSION

Field collection and screening

Approximately 10% of field-collected insects from both New York and Maryland survived doses of 30 μg dieldrin in jars. From this apparently high frequency of resistance, strains surviving 300 μg in scintillation vials were obtained after two to four generations of selection.

Characterization and chromosomal localization

Dose response lines for the presumed homozygous resistant (*R/R*) and susceptible (*S/S*) strains as well as the putative heterozygotes (*R/S*) are shown in Fig. 1. As a phenotype resistance is essentially recessive, i.e. a dose of 30 μg per scintillation vial reliably discriminates *RR* individuals from *RS* and *SS*, whereas a dose of 0.5 μg only partially discriminates *SS* individuals from *RS* and *RR*. Thus the former diagnostic dose was usually used to score for resistance. This dose/response pattern was assumed to result from a single gene as confirmed by subsequent genetic analysis described below.

Chromosomal localization

In order to test for sex linkage, *R/R* females (hypothetically *XR/XR* were crossed to susceptible males (*XS/Y*) and the male and female progeny screened separately at 0.5 μg dieldrin. If resistance is sex linked, fewer of the resulting hemizygous resistant males (*XR/Y*) would die than the heterozygous females (*XR/XS*). However, mortality was greater in males (68%, number tested =178) than females (53%, n=144).

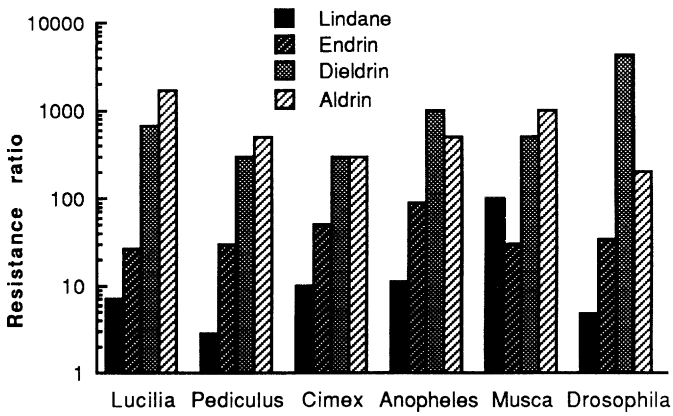


Figure 2. Cross-resistance spectrum of cyclodiene resistant insects to dieldrin, aldrin, endrin and lindane. Data except for *Drosophila melanogaster* are from Oppenoorth (1985).

Table 1. Resistance of isogenic dieldrin-resistant (MD-BC5) and susceptible (Oregon) *Drosophila melanogaster* (see ffrench-Constant and Roush, in press).

INSECTICIDE	RESISTANCE RATIO [LC50(R)/LC50(S)]
Propoxur	0.2
Malathion	0.6
DDT	1.0
Picrotoxin	$2x10^5$
Lindane	5.0
Dieldrin	4270
Aldrin	194
Endrin	33

To test for association with chromosome II, male *BLL/R* heterozygotes were crossed to Oregon R (*S/S*). The *Bl L* and wild type progeny were then screened separately at 0.5 μg. Results were again the opposite of expected, with higher mortality of the *R/S* flies (60%, n=186) than the hypothetical *Bl L /S* flies (55%, n=l00). For chromosome III, *R/R* flies were crossed to *Ly Sb/Lvm,* their *Ly Sb /R* progeny then backcrossed to *R R* and the progeny tested at 30 μg. About 50% (n=1057) of the progeny survived, whereas all the *LY Sb /R* F_1 individuals died at this discriminating dose. Of the 1057 flies scored, 167 recombinants were recorded, producing a map distance distal from lyra (map unit 40.5) of 15.8, thus placing the resistance gene at approximately map unit 25 on the left arm of chromosome three (ffrench-Constant et al. in press). These data strongly suggest that a single gene is responsible for resistance.

As resistance is recessive, hemizygous *R/Df* flies should display resistance equivalent to *RR*,. whereas *R/TM3* flies are *R/S* and should die at 30 μg dieldrin. For both New York and Maryland flies, only one deficiency (Df(3L)29A6, spanning 66F5-67Bl) showed high

levels of resistance in *R/Df* progeny (e.g. 83% survival in crosses with *MD-RR*), whereas all other *R/Df* and *R/TM3* progeny for the remaining deficiencies showed only 0-2% survival. We assume from these results that dieldrin resistance in the Maryland and New York strains is allelic and complimentation tests will be carried out to confirm this. The overlapping Df(3L)ACl (67A-67D) does not uncover resistance, thus localizing the resistance gene to 66F (the region of Df(3L)29A6 not overlapped by Df(3L)ACl) (ffrench-Constant and Roush in press). The salient breakpoints isolating this region have subsequently been confirmed by RJM as 66F1-2 for the distal break of Df(3L)29A6 and 67A1-2 for the distal break of Df(3L)ACl. This corresponds closely with their initial description by Leicht and Bonner (1988).

Cross-resistance

Following backcrossing of the resistance gene into the Oregon R background, resistance in the *MD-BC5* strain to propoxur, malathion or DDT was lower than or equal to that of Oregon R. In contrast, resistance was shown to the GABA antagonists tested, including lindane(γ HCH) and picrotoxinin, and very high to the cyclodienes dieldrin, aldrin and endrin (Table 1; ffrench-Constant and Roush, in press). Resistance ratios from these data are similar to those for other insects showing cyclodiene resistance (Fig. 2). Cyclodiene-resistant animals typically show cross resistance to the closely related lindane (γ HCH) and to the GABA antagonist picrotoxinin (Kadous et al. 1983, Tanaka 1987, Yarbrough et al. 1986).

Cosmid walk and microinjection

A phage clone (lambda 121) in Charon 4a was obtained and confirmed to hybridize *in situ* to 66F1-2. This was then used to initiate a cosmid walk (Bender et al. 1983). Four overlapping cosmids spanning approximately 100 kb of the region have been isolated. Their positions as indicated by *in situ* hybridization are shown in Fig. 3. *In situ* hybridization of the fourth cosmid to Df(3L)ACl heterozygotes reveals labelling of both homologs and thus the distal breakpoint of Df(3L)ACl has not yet been reached.

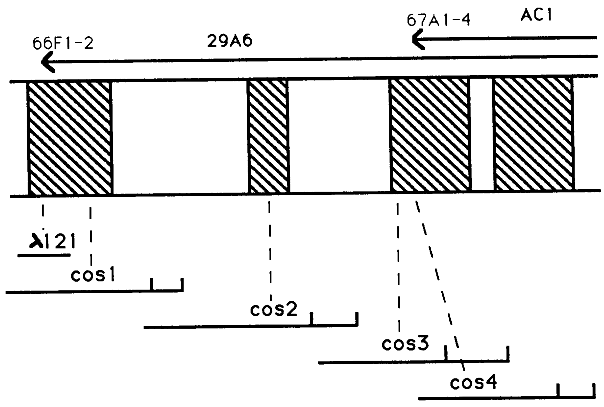


Figure 3. Diagram representing the cytological location 66F on the polytene chromosome. Arrows above the region represent the extent of the deficiency breakpoints isolating the resistance gene and lines below the locations of the original phage (lambda 121) and cosmids 1-4 of the walk following *in situ* hybridization.

Cosmids isolated from the region are currently being microinjected into w; Δ2, 3 Sb/TM6 embryos (Δ2, 3 provides an internal source of transposase). Following successful transformation, as denoted by the detection of w+ G_1 flies and determination of which chromosome carries the insert, these transformants as putative carriers of the susceptible gene (w+S), will be repeatedly backcrossed (Fig. 4) to *R/R* flies. Putative *w+S; R/R* flies will be screened at 30 μg in order to establish which cosmid contains a copy of the dominant

susceptible gene and thus confers susceptibility on an *R/R* chromosomal background.

Successful transformation of one of the cosmids has been achieved at a frequency of approximately 1% of single families established from G_0(microinjected) survivors. Further microinjections and crosses are in progress to determine which of the cosmids if any contains the gene.

Further attempts to find which cosmid carries the susceptible gene are being made by generating new deficiencies using gamma irradiation. Deficiencies uncovering resistance can be isolated in an Fl screen, thus facilitating the examination of a large number of flies. Following isolation of new deficiencies uncovering the resistance gene their breakpoints will be examined to see if they lie within the region containing the resistance gene.

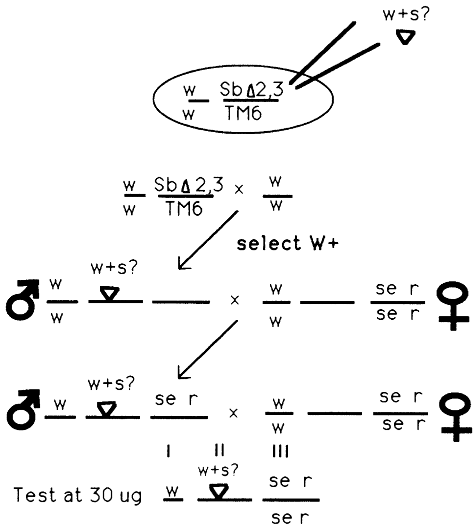


Figure 4. Crossing scheme used for micro-injection of cosmids and backcrossing of putative susceptible gene (S?) carrying inserts to a marked *R/R* strain. The white gene (w^+) is used as an eye color marker and $\Delta 2,3$ acts as an endogenous source of transposase in the injected embryos. Resulting progeny are screened at 30 µg dieldrin to establish if susceptibility has been rescued.

CONCLUSIONS

The finding of high levels of cyclodiene resistance in *D melanogaster* suggests that resistant mutants can be recovered from field populations of this insect. Isolation of dieldrin resistance at readily detectable frequencies from the field may either suggest that resistance is not associated with any major fitness disadvantages and has been maintained since withdrawal of cyclodienes from use in the 1960's or that resistance is maintained by selection with endosulfan, a cyclodiene that is still in use and to which there are typically low levels of cross-resistance. Recovery of resistant strains from the field shows that the problem of absence of selection pressure in the field anticipated by Wilson (1988) in the use of *D. melanogaster* as a model for research on the molecular genetics of resistance can be overcome, at least for some insecticides. Thus, it will now be possible to compare directly resistant populations selected or generated in the laboratory with resistance that has evolved in the field.

The evidence presented here that resistance is due to a single major gene on the left arm of chromosome III is consistent with predictions that can be made from homologies with *Lucilia cuprina* and *Musca domestica* where a gene for cyclodiene resistance is found on chromosomes five and four, respectively (Foster et al. 1981). These are the chromosomes homologous to chromosome III in *D. melanogaster*. That the levels of resistance to cyclodienes are similar to those found in other cyclodiene resistant insects and that the *R/R* flies exhibit cross-resistance to lindane and picrotoxinin also strongly support the view that this gene is homologous to that in other insects.

The exact nature of the resistance mechanism remains uncertain, and thus also the product of the resistance gene. Resistance could be caused either by an insensitive GABA receptor or possibly a reduction in number of receptors (Tanaka 1987). However the ability in *D. melanogaster* to clone genes of known location but with unknown products via chromosomal walking should allow the elucidation of the function of this gene.

ACKNOWLEDGMENTS

We thank S. Rosensweig and D. Mortlock for excellent technical assistance, J. Werner for microinjections, M.B. Davis, M. Becker and C. Schaffer for useful advice and assistance, and G. Collier for provision of the phage clone. R.H. ffrench-Constant was supported by a postdoctoral fellowship from Merck Sharp and Dohme Research Laboratories. The Rhone-Poulenc Agrochemical Company provided additional support for technical assistance and supplies.

REFERENCES

Bender, W., Spierer, P. and Hogness, D.S. 1983. Chromosomal walking and jumping to isolate DNA from the *Ace* and *rosy* loci and the bithorax complex in *Drosophila melanogaster*. J. Mol. Biol. 168: 17-33.

Berge, J.B. and Fournier, D. 1988. Advances in molecular genetics of acetylcholinesterase insensitivity in insecticide-resistant insects. p. 461 in: "Procedings XVIII International Congress of Entomology." Vancouver.

Dillela, A.G. and Woo S.L.C. 1985. Cosmid cloning of genomic DNA. Focus (Bethesda Research Laboratories) Vol. 7(2): 5.

Eldefrawi, A.T. and Eldefrawi, M.E. 1987. Receptors for γ-aminobutyric acid and voltage-dependent chloride channels as targets for drugs and toxicants. FASEB J. 1: 262-271.

Feyereisen, R., Koener, J.F., Farnsworth, D.E. and. Nebert, D.W. 1989. Isolation and sequence of cDNA encoding a cytochrome P-450 from an insecticide-resistant strain of the house fly, *Musca domestica*. Proc. Natl. Acad. Sci. USA. 86: 1465-1469.

ffrench-Constant, R.H. and Roush, R.T. 1990. Gene mapping and cross-resistance in cyclodiene insecticide resistant *Drosophila melanogaster*. Genebical Research. In press.

ffrench-Constant, R.H., Roush, R.T., Mortlock, D. and Dively, G.P. 1990. Isolation of dieldrin resistance from field populations of *Drosophila melanogaster*. J. Econ. Entomol. In press.

Field, L.M., Devonshire, A.L. and Forde, B.G. 1988. Molecular evidence that insecticide resistance in peach-potato aphids (*Myzus persicae*) results from amplification of an esterase gene. Biochem. J. 251: 309-312.

Foster, G.G., Whitten, M.J., Konovalov, C., Arnold, J.T.A. and Maffi, G. 1981. Autosomal genetic maps of the Australian sheep blowfly, *Lucilia cuprina dorsalis* R-D. and possible correlations with the linkage maps of *Musca domestica* and *Drosophila melanogaster*. Genet. Res. 37: 55-69.

Georghiou, G.P. 1986. The magnitude of the resistance problem. pp. 14-43 in: "Pesticide resistance: Strategies and tactics for management." National Academy of Sciences, ed. National Academy Press: Washington, D.C.

Houpt, D.R., Pursey, J.C. and Morton, R.A. 1988. Genes controlling malathion resistance in a laboratory selected population of *Drosophila melanogaster*. Genome 30: 844-853.

Kadous, A.A., Ghiasuddin, S.M., Matsumura, F., Scott, J.G. and Tanaka, K. 1983. Difference in the picrotoxinin receptor between the cyclodiene resistant and susceptible strains of the German cockroach. Pestic. Biochem. Physiol. 19: 157-166.

Kasbekar, D.P. and Hall, L.M. 1988. A *Drosophila* mutation that reduces sodium channel number confers resistance to pyrethroid insecticides. Pestic. Biochem. Physiol. 32: 135-145.

Leicht, B.G. and Bonner, J.J. 1988. Genetic analysis of chromosomal region 67A-D of *Drosophila melanogaster*. Genetics. 119: 579-593.

Morton, R.A. and Singh, R.S. 1982. The association between malathion resistance and acetylcholinesterase in *Drosophila melanogaster*. Biochem. Gen. 20: 179-198.

Oppenoorth, F.J. 1985. Biochemistry and genetics of insecticide resistance. pp. 731-773 in: "Comprehensive Insect Physiology, Biochemistry, and Pharmacology." vol. 12. G.A. Kerkut and L.I. Gilbert, ed. Pergamon, New York.

Pardue, M.L. 1986. *In situ* hybridization to DNA of chromosomes and nuclei. pp. 111-137 in: "*Drosophila*, A Practical Approach". D.B. Roberts, ed. IRL Press, Oxford, England.

Roush, R.T. and Daly, J. 1990. The role of population genetics in resistance research and management. in: "Pesticide Resistance in Arthropods." Roush, R.T. and Tabashnik, B.E., eds. Chapman and Hall, New York. in press.

Roush, R.T. and McKenzie, J.A. 1987. Ecological genetics of insecticide and acaricide resistance. Ann. Rev. Entomol. 32: 361-380.

Schofield, P.R., Darlison, M.G., Fugita, N., Burt, D.R., Stephenson, F.A., Rodriguez, H., Rhee, L.M., Ramachandran, J., Reale, V., Glencorse, T.A., Seeburg, P. H. and Barnard, E. A. 1987. Sequence and functional expression of the $GABA_A$ receptor shows a ligand-gated receptor super-family. Nature 328: 221-227.

Soderlund, D.M. and Bloomquist, J.R. 1990. Molecular mechanisms of insecticide resistance. in: "Pesticide Resistance in Arthropods." Roush, R.T. and Tabashnik, B.E., eds. Chapman and Hall, New York. in press.

Tanaka, K. 1987. Mode of action of compounds acting at inhibitory synapse. J. Pestic. Sci. 12: 549-560.

Waters, L.C. and. Nix, C.E. 1988. Regulation of insecticide resistance-related cytochrome P-450 expression in *Drosoohila melanogaster*. Pestic. Biochem. Physiol. 30: 214-227.

Wilson, T.G. 1988. *Drosoohila melanogaster*: A model insect for insecticide resistance studies. J. Econ. Entomol. 81: 22-27.

Wilson, T.G. and Fabian, J. 1986. A *Drosophila melanogaster* mutant resistant to a chemical analog of juvenile hormone. Developmental Biology 118: 190-201.

Yarbrough, J.D., Roush, R.T.,. Bonner, J.C and Wise, D.A. 1986. Monogenic inheritance of cyclodiene resistance in mosquito fish, *Gambusia affinis*. Experientia 42: 851-853.

CLONING, EXPRESSION AND BIOLOGICAL ACTIVITY OF THE JUVENILE HORMONE ESTERASE FROM *Heliothis virescens*

B. Hammock[1], V. Wroblewski[1], L. Harshman[1], T. Hanzlik[1], S. Maeda[2], M. Philpott[1], B. Bonning[3], and R. Possee[3]

[1]Departments of Entomology and Environmental Toxicology and
[2]Department of Entomology, University of California, Davis, 95616, U.S.A.
[3]NERC Institute of Virology and Environmental Microbiology, Mansfield Road, Oxford, OX1 3SR, U.K.

INTRODUCTION

One of the more exciting trends in science is the increasingly short space of time between many fundamental discoveries and their impact on applied science. In turn, applied problems increasingly can be addressed directly by fundamental approaches. While solving such applied problems one often sees an advancement of the fundamental field as well. These trends certainly are illustrated by our recent work on the juvenile hormone esterases (JHE's) of lepidopterous larvae. Expression of this enzyme in several baculovirus systems may have impact on pest control and viral ecology, but certainly this work will advance our fundamental knowledge of insect endocrinology.

Some years ago the hypothesis was advanced that juvenile hormone regulation in some insects could be accomplished by a combined decrease in biosynthesis and an increase in degradation (Hammock, 1985). Although regulation of chemical mediators by degradation is common with neurotransmitters and neurohormones, variations in the rates of degradation have not been widely considered as significant with epithelial hormones.

In insects, which must effect major changes in hormone titer in short periods of time, it is not surprising that regulation of both biosynthesis and degradation should be employed. With juvenile hormone this possibility was certainly suggested in *Manduca sexta* when Weirich et al. (1973) reported a correlation between the ability of the hemolymph to hydrolyze the methyl ester of JH and the reduction of JH titer. Experimental support for this hypothesis resulted first from the demonstration that inhibition of this juvenile hormone esterase (JHE) with selective organophosphates gave developmental effects similar to the application of juvenile hormone (Sparks and Hammock, 1980). The case was strengthened when it was found that a novel group of highly selective "transition state mimics" of JHE which resembled JH could inhibit the enzyme and result in similar giant larvae (Hammock et al., 1984). This hypothetical role of JHE was further supported by the demonstration that inhibition of JHE blocked the complete clearance of JH when monitored by GLC-MS. This work demonstrated that very little JH was needed to block metamorphosis and provided an explanation for why reduced biosynthesis alone could not clear JH in preparation for pupation (Jones et al., 1989).

The alternate approach to testing this hypothesis of JHE involvement in the initiation of metamorphosis is to demonstrate anti-JH effects elicited by the precocious appearance of JHE. In this manuscript we summarize recent work from our laboratories on the biochemistry and molecular biology of JHE leading to such experiments. It is hoped that the results of this work will help to elucidate mechanisms of JH regulation in other systems. However, it must be cautioned that the regulatory mechanisms are likely to be very different in other species.

ISOLATION OF ENZYME AND MESSAGE

Since JHE is a low abundance protein present in a limited tissue source, the development of a highly efficient affinity purification system for the enzyme was critical. The trifluoromethylketone column resulting from this work proved very efficient for purifying the JHE from the hemolymph of a variety of lepidopterous larvae if the general esterases had been previously inhibited (Abdel-Aal and Hammock, 1986; Hammock et al., 1988). In fact this column appears generally applicable to the purification of esterases from many species.

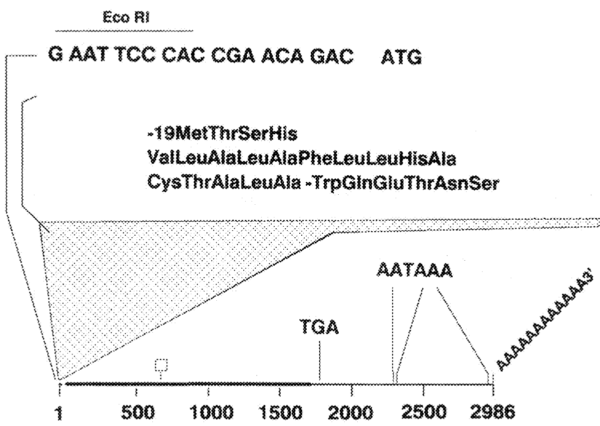


Fig. 1. Key characteristics of the sequence of a 2986 bp JHE message from *H. virescens*. Following the *Eco*RI site is a consensus sequence for an insect ribosome binding site (top). Following the first ATG there is a putative 19 amino acid leader sequence before a deduced N-terminal sequence starting with TrpGln which matches closely the sequence determined by Edman degradation. The coding region is shown by the bold line and the hypothetical catalytic serine by a square. Following the stop codon is a long noncoding region shown by a light line with 3 polyadenylation sites. The numbers at the bottom refer to the bases of the cDNA.

The resulting pure proteins allowed the determination of N-terminal sequences and the development of antibodies for the JHE's from *Heliothis virescens, M. sexta,* and *Trichoplusia ni*. These probes were used to isolate three apparently full length messages from a fat body cDNA library of *H. virescens.*

The three clones (3hv1, 3hv16 and 3hv21) had similar sequences. As shown in Fig. 1 one of the clones (3hv21) had an insert of 2989 bp which approximates the length of the putative JHE message detected by Northern blot. The derived amino acid sequence from the 1714 bp open reading frame predicts a 563 amino acid 61 kDa protein which agrees well with SDS-PAGE analysis of the hemolymph protein (Hanzlik et al., 1989).

SEQUENCE ANALYSIS

Comparison of these JHE sequences with other known esterases may provide some insight into the catalytic mechanism of esterases. Over 20 percent homology was observed among JHE and several other esterases suggesting that these enzymes arise from a common ancestral enzyme. The region of greatest similarity lies near the N-terminal end of the protein (Fig. 2).

In the early days of X-ray analysis of protein crystals Blow and his associates developed an exciting hypothesis to explain the high reactivity of the catalytic serine of proteases based on proton transfer from serine through histidine to aspartic acid (Blow, 1976). With some adjustment this catalytic triad theory still seems applicable to protease action (Smith et al., 1989). Since esterases are known to have a similar catalytically active serine, it was not surprising that the triad was also assumed to exist in esterases. However,

sequence analysis of a variety of esterases indicated that either esterases have a radically different folding pattern than proteases or that a different catalytic system was involved. A single catalytically important serine is present in the Gly-X-Ser-X-Gly sequence seen in proteases, but if the later hypothesis is correct the catalytic triad is replaced by highly conserved regions corresponding to Asp 102 and His 57 of chymotrypsin. The acid motif of most esterases would accommodate either aspartic or glutamic acid acting as the final proton acceptor of a catalytic triad. However, JHE has only aspartic acid. The situation is more complex if one tries to find a basic amino acid substitute for the histidine 57. The Arg-Phe region of esterases corresponds to this His motif of proteases. Although Arg could orient and activate a water molecule involved in a proton transfer reaction, polarize the ester carbonyl, or even activate serine directly, it is difficult to envision how it could play an analogous role to that hypothesized for His in proteases (Hanzlik et al., 1989).

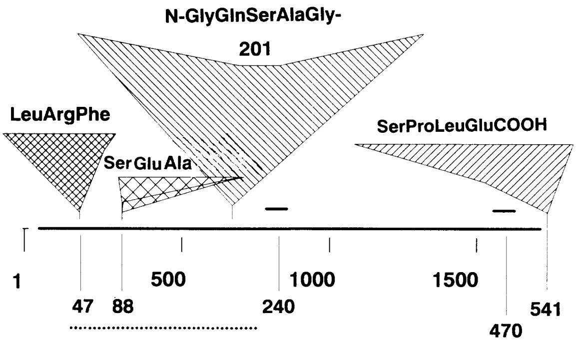


Fig. 2. Deduced amino acid sequences of putative catalytic motifs of the 1.7 kbp translated region of the JHE cDNA. In addition to the C-terminal sequence, three sequences which may correspond to the catalytic triad of serine proteases are shown. The two small solid lines show polar regions of acetylcholine esterase thought to be involved in substrate binding which are nonpolar in JHE. The dotted line indicates the region of greatest homology with the other serine esterases and thyroglobulin. The upper numbers indicate base number and the lower ones refer to amino acid number.

There are a variety of other highly conserved motifs in esterases which may be catalytically important. However, X-ray studies and site directed mutagenicity are needed to support hypotheses on the catalytic mechanism of esterases. It will be interesting to see if the highly conserved Arg-Phe region is catalytically important.

GENE REGULATION

The regulation of JHE appears complex. Its appearance in the hemolymph early in the last larval stadium appears to be under a different control system than its appearance during the prepupal period (Hammock, 1985). Further complexities exist when one considers the vastly different activities present in tissues and hemolymph in the pupa, its low apparently constitutive appearance in tissues in early instars and its brief appearance in the hemolymph during larval molts in *T. ni*. Our understanding of this regulation is at best superficial. However, an 851 bp *Bam*HI fragment from the 5' end of the *H. virescens* JHE clone has been used to detect both RNA and DNA from several species. Hopefully this and other probes will accelerate aspects of the above work on JHE regulation in a number of laboratories.

When this fragment was used to probe RNA isolated from larvae of *H. virescens* of different ages, a 3 kbp fragment was always found by Northern analysis. The message was not detected in third stadium larvae when JHE expression is very low, but was detected in the last larval stadium. On day 2 of this stadium, mRNA levels in the integument were 3 times higher than in the fat body. However, two days later when hemolymph activity of JHE reaches peak levels, the JHE mRNA detected in the fat body increased 9 times while

the levels in the epidermis decreased slightly. These data suggest that JHE can be differentially regulated in tissues and that the majority of the JHE in peak hemolymph levels comes from the fat body. The level of message and catalytic activity in both tissues could be dramatically increased by treatment of whole insects with the juvenoid epofenonane.

Since both message and protein levels (determined by Western blot) correlate with the catalytic activity of JHE, it appears that transcriptional control is very important in regulating JHE expression. However, it also seems clear that the ability of a tissue to hydrolyze JH is controlled by a variety of factors including the stability of the message and protein (Wroblewski et al., 1989).

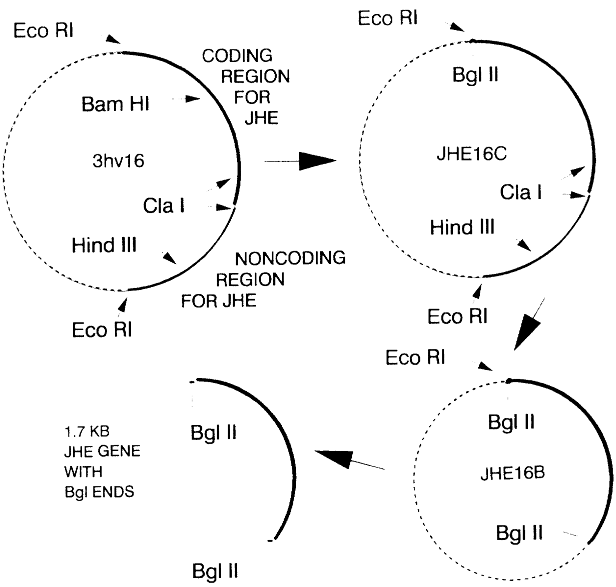


Fig. 3. Plasmids used in the isolation of 1.7 kbp coding fragment of the juvenile hormone esterase from *H. virescens*. The Bluescript plasmid is represented by the dotted line and the 3' noncoding and coding regions of the JHE gene by increasingly heavier lines. Plasmid 3hv16 is described by Hanzlik et al. (1989). Plasmid JHE16C was modified with *Bgl*II linkers flanking an *Eco*RI site at the 5' end of the gene. Plasmid JHE16B has the DNA from the *Cla*I site downstream from the natural stop codon through the *Cla*I site in the polylinker removed and replaced by a *Bgl*II linker.

BACULOVIRUS EXPRESSION OF JHE

The development of an expression system for JHE allows one to address a variety of problems. It obviously allows one to test if the isolated sequence is catalytically active as well as facilitating the production of large amounts of enzyme for biological and pharmacokinetic studies. Since the cell lines used are of lepidopterous origin, it is likely that expression will shed some light on the natural processes of post translational modification and transport of the enzyme. Finally, from a practical perspective, it may lead to the production of baculoviruses of improved characteristics. Toward these ends we are working on the expression of full and partial sequences of JHE in baculovirus systems from *Autographa californica* and from *Bombyx mori*.

Expression with NPV of A. californica

For expression in this system a transfer vector (pAcRP23) was used which had a *Bam*HI cloning site (Matsura et al., 1987). This presented some difficulty since the JHE plasmid used had an internal *Bam*HI site as discussed above. Thus, as shown in Fig. 4 the 3hv16 plasmid was linearized by partial digestion with *Eco*RI. The ends of the purified DNA were filled in with the Klenow fragment of *E. coli* DNA polymerase, *Bgl*II linkers

added, and the DNA relegated to give a 5' *Eco*RI site flanked by *Bgl*II sites. This plasmid (JHE16C) was grown and DNA again isolated. A partial digestion with *Cla*I removed most of the 3' noncoding region while retaining the natural stop codon and the full 1.7 kbp coding region. The above process of adding *Bgl*II linkers at the 3' end of the JHE gene was used to give plasmid JHE16B (Fig. 3). *Bgl*II digestion of this plasmid yielded the 1.7 kbp fragment which could be easily cloned into the *Bam*HI site of pAcRP23 to yield pAcRP23.JHE. This recombinant transfer vector was co-precipitated with wild type virus on to cells of *Spodoptera frugiperda* (IPLB Sf 21) and a recombinant virus isolated by repeated plaque purification. In the virus AcRP23.JHE the JHE gene is under the control of the polyhedrion promoter and the polyadenylation site of the polyhedrion gene is used. This results in high levels of expression very late in infection.

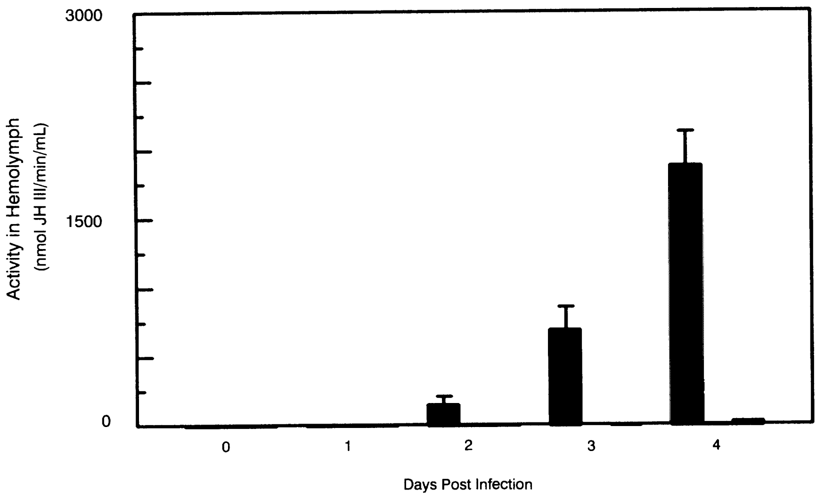


Fig. 4. Expression of JHE activity *in vivo* by a recombinant BmNPV virus. Larvae of ***Bombyx mori*** from the second day of the fifth instar were injected with 1.5 x 10^7 PFU's of the recombinant virus, 3hvl-1, that contained cDNA encoding JHE. Their hemolymph was then assayed for JH hydrolysis at one day intervals until death which occurred on the fifth day post infection (solid bar). The activities in the hemolymph of larvae injected with a recombinant that did not express JHE and sham injected larvae (right hatch) were also measured. No differences in morphology or weight gain between the control larvae and those injected with 3hvl-1 were noted. Data are the result of measurements from triplicate larvae. Data for larvae infected with the control recombinant virus were near zero and thus not apparent on the graph.

Since the ester of JH is so stable to most esterases, it was possible to detect high JHE activity even in crude transfections in the presence of high general carboxylesterase activity in fetal calf serum (FCS). It also was possible to monitor the JHE activity in agar associated with JHE producing plaques while *S. frugiperda* cells did not produce detectable JHE activity even when infected with several control viruses. Hydrolysis of JH by the crude culture medium from infected cells was found to be linearly dependent on protein concentration and time over a wide range of conditions. The expressed enzyme also was stable to repeated freeze thaw cycles, storage for several days at room temperature, and to a variety of denaturing reagents. This sensitive assay for JHE (Hammock and Sparks 1977) greatly simplifies the isolation of recombinant virus.

As expected from the leader sequence, the JHE is rapidly exported into the medium of infected cells. In all cases over 90 percent of the catalytic activity was found to be extracellular. Following infection in the presence of 5% FCS good production of JHE could be obtained in media lacking FCS and SDS-PAGE of the resulting media revealed JHE as the predominant protein as well as a major cellular protein. Depending on culture conditions up to 250 nmol/min of JH could be hydrolyzed per ml of culture media which is far higher than maximal rates obtained in the hemolymph of *H. virescens*.

When infected with the recombinant virus either orally or by injection, JHE activity is detected in the hemolymph of *T. ni* at levels far higher than normally present in early instar larvae. The levels are similar to those seen in normal early instar larvae at ecdysis. It is thought that the expression of JHE at this time results in a transient decrease in JH which reduces feeding behavior (Hanzlik and Hammock, 1988). However, the expressed levels are only about 10% of those normally seen in the last larval stadium of *T. ni* or *H. virescens*.

Expression with NPV of B. mori

For expression in the *B. mori* system, the full 3 kbp insert from the 3hv1 clone was used. Thus, it was possible for one of the 3 natural polyadenylation sites as well as the polyadenylation site of the polyhedron gene to be used. The JHE gene was removed from a Bluescript plasmid with *Eco*RI and cloned into an *Eco*RI site of the transfer vector pBE274. Cotransfection was carried out in a similar manner to that described above according to Maeda (1989). Expression *in vitro* was comparable to that observed in the *A. californica* system with the majority of the catalytic activity again detected in the media. However, expression *in vivo* with the *B. mori* system was very high. When fifth instar larvae of *B. mori* were injected with the recombinant virus, JHE activity could be detected as soon as two days post infection with activity increasing to protein levels corresponding to nearly 1 mg/ml in the hemolymph at 4 days post infection (Fig. 4). Death occurred with the recombinant virus as well as wild-type virus and a virus containing a defective JHE gene five days post infection. With both systems *in vivo* and *in vitro*, the protein purified by affinity chromatography was similar to that isolated from the hemolymph when analyzed by SDS-PAGE and Western blotting.

Biological JHE Effects

When JHE isolated by affinity chromatography from the blood of *M. sexta* or culture media of NPV infected cells of *S. frugiperda* was injected into third stadium larvae of *M. sexta*, the larvae turned black at the following molt in a dose dependent fashion. The effects of this injection of picomole amounts of JHE could be reversed by the application of a juvenoid resistant to ester hydrolysis. Thus, the natural and expressed enzymes have clear anti-juvenile hormone effects. However, we were unable to generate precocious pupae using the enzyme isolated from *M. sexta* possibly due to its short half life *in vivo* (Philpott and Hammock, 1989).

Infection of first stadium larvae of *T. ni* with AcRP23.JHE resulted in a reduction in feeding and weight gain. Although this reduction in feeding was not noticeable compared to uninfected control larvae or larvae infected with a virus expressing the gp 120 coat protein of HIV until 3-4 days post infection, the difference in size of the larvae by day 5 was dramatic. The size of larvae fed AcRP23.JHE, but not control viruses, was significantly increased by application of juvenoids. In contrast, no morphological changes have been observed in larvae of *B. mori* similarly infected with the recombinant NPV.

From the perspective of practical application, the lack of significant *in vivo* effects in *B. mori* are disappointing, but the effects in *T. ni* are very encouraging. Based on the biology of JHE it is likely that the insect has a mechanism which allows the rapid turn over of the enzyme. This may explain why *in vivo* expression of a JHE from a closely related species is so much lower than *in vitro* expression. The fact that low apparent expression of JHE under the control of a very late promoter can apparently overcome biosynthesis of JH and result in a biological effect indicates that higher levels of expression under an earlier promoter could improve the efficacy of viral insecticides.

The results from the above expression systems confirm that the isolated cDNA clones from *H. virescens* do code for JHE, that JHE may be a valuable reporter enzyme for studying promoter activity, and that expressed JHE can act as a biochemical anti-JH. Certainly the very sensitive catalytic assay for JHE coupled with sensitive immunochemical and hybridization assays will simplify detection of insects infected with the recombinant virus in ecological studies. It also is hoped that several approaches can be taken to increase the biological activity of the engineered viruses. Such work could involve the alteration of the DNA coding for JHE such that the message and/or expressed JHE protein is resistant to degradation in target insects as well as the examination of different viral promoter systems. However, the greatest value of the sequence for JHE will be as a probe in elucidating fundamental mechanisms of developmental regulation.

ACKNOWLEDGMENTS

This work was supported by grants from NSF (DCB-8518697) and USDA (85-CRCR-1-11715). The current address of V.W. is Drug Disposition Research Division, Lilly Research Laboratories, Eli Lilly & Co., Indianapolis, IN 46285. B.H. is a Burroughs Wellcome Toxicology Scholar and thanks the Fogarty Foundation (NIH) for supporting his sabbatical leave with R.P. B.H. also thanks the powers that be for not having things fall on him during the earthquake that occurred while typing the manuscript.

REFERENCES

Abdel-Aal, Y.A.I. and Hammock, B.D. 1986. Transition state analogs as ligands for affinity purification of juvenile hormone esterase. Science 233: 1073-1076.

Blow, D.M. 1976. Structure and mechanism of chymotrypsin. Accounts Chem. Res. 9: 145-152.

Hammock, B.D. 1985. Regulation of juvenile hormone titer: degradation. pp. 431-472 in: "Comprehensive Insect Physiology Biochemistry and Pharmacology." Kerkut, G.A. and Gilbert, L.I. eds. Pergamon Press, N.Y.

Hammock, B.D. and Sparks, T.C. 1977. A rapid assay for insect juvenile hormone esterase activity. Anal. Biochem. 82: 573-579.

Hammock, B.D., Abdel-Aal, Y.A.I., Mullin, C.A., Hanzlik, T.N. and Roe, R.M. 1984. Substituted thiotrifluoropropanones as potent selective inhibitors of juvenile hormone esterase. Pestic. Biochem. Physiol. 22: 209-223.

Hammock, B.D., Harshman, L.G., Philpott, M.L., Szekacs, A., Ottea, J.A., Newitt, R.A., Wroblewski, V.J., Halarnkar, P.P. and Hanzlik, T.N. 1988. Strategies for the discovery of insect control agents: Exploitation of biomechanisms regulating insect development. pp. 137-173 in: "Biomechanisms Regulating Growth and Development." USDA Beltsville Symposia in Agricultural Research, Vol.12. Steffens, G.L. and Rumsey, T.S. eds. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Hammock, B. D., Bonning, B., Possee, R. D., Hanzlik, T. N. and Maeda, S. 1990. Expression and effects of the juvenile hormone esterase in a baculovirus vector. Nature in press.

Hanzlik, T.N. and Hammock, B.D. 1988. Characterization of juvenile hormone hydrolysis in early larval development of *Trichoplusia ni*. Arch. Insect Biochem. Physiol. 9: 135-156.

Hanzlik, T.N., Abdel-Aal, Y.A.I., Harshman, L.G. and Hammock, B.D. 1989. Isolation and sequencing of cDNA clones coding for juvenile hormone esterase from *Heliothis virescens*. J. Biol. Chem. 264: 12419-12425.

Jones, G., Hanzlik, T., Hammock, B.D., Schooley, D.A., Miller, C.A., Tsai, L.W. and Baker, F.C. 1989. The juvenile hormone titre during the penultimate and ultimate larval stadia of *Trichoplusia ni*. J. Ins. Physiol. in press.

Maeda, S. 1989. Gene transfer vectors of a baculovirus, *Bombyx mori* nuclear polyhedrosis virus, and their use for expression of foreign genes in insect cells. pp. 167-181 in: "Invertebrate cell system applications." Vol. I. Mitsuhashi, J. ed. CRC Press, Boca Raton, Fl. .

Matsuura, Y., Possee, R.D., Overton, H.A. and Bishop, H.L. 1987. Baculovirus expression vectors: the requirements for high level expression of proteins, including glycoproteins. J. Gen. Virol. 68: 1233-1250.

Philpott, M.L. and Hammock, B.D. 1989. Juvenile hormone esterase is a biochemical anti-juvenile hormone agent. Insect Biochem. in press.

Smith, S.O., Farr-Jones, S., Griffin, R.G., and Bachovchin, W.W. 1989. Crystal vs. solution structures of enzymes NMR spectroscopy of a crystalline serine protease. Science 244: 961-964.

Sparks, T.C. and Hammock, B.D. 1980. Comparative inhibition of the juvenile hormone esterases from *Trichoplusia ni, Tenebrio monitor,* and *Musca domestica*. Pestic. Biochem. Physiol. 14: 290-302.

Weirich, G., Wren, J. and Siddall, J.B. 1973. Developmental changes of the juvenile hormone esterase activity in haemolymph of the tobacco hornworm, *Manduca sexta*. Insect Biochem. 3: 397-407.

Wroblewski, V.J., Harshman, L.G., Hanzlik, T.N. and Hammock, B.D. 1989. Regulation of juvenile hormone esterase gene expression in the tobacco budworm, (*Heliothis virescens*). Arch. Biochem. Biophys. in press.

CLONING AND CHARACTERISATION OF A MOSQUITO ACETYLCHOLINESTERASE GENE

Colin A. Malcolm[1] and Lucinda M.C. Hall[2]

[1]School of Biological Sciences, Queen Mary and Westfield College, University of London, Mile End Road, LONDON E1 4NS.
[2]Department of Medical Microbiology, The London Hospital Medical College, The University of London, Turner Street, LONDON E1 2AD.

INTRODUCTION

Acetylcholinesterase (AChE, EC 3.1.1.7), a serine hydrolase, catalyzes the breakdown of the neurotransmitter acetylcholine into acetate and choline. This involves the formation of a substrate enzyme complex, followed by acetylation of the hydroxyl group of the amino acid serine, present within the esteratic site, and finally deacetylation. AChE is the target site for carbamate and organophosphate (OP) insecticides. These compounds react in an analogous way to acetylcholine, forming a complex, then respectively carbamylating or phosphorylating the enzyme. The dephosphorylating and decarbamylating steps are however very slow thus inhibiting the enzyme. (O'Brian 1976).

In a number of pest species altered AChEs have been found with a reduced sensitivity to inhibition by OPs and carbamates, which results in a broad spectrum resistance to these two groups of insecticides. In several species, insensitive AChEs have been found which show no marked difference in enzymatic activity compared to sensitive alleles, whereas in other insects a reduction has been found. In some studies a number of different mutant AChEs have been identified for the same species with varying degrees of insensitivity (See Oppenoorth 1985 for review). In mosquitoes, insensitive AChE has been identified in seven species, *Anopheles albimanus*, *An. atroparvus*, *An. sacharovi*, *An. nigerimus*, *Culex pipiens*, *C. tritaeniorhynchus* and *C. quinquefasciatus* (cited in ffrench-Constant and Bonning, 1989).

Only one type of cholinesterase has been found in insects in contrast to the two in vertebrates, which are distinguished by their substrate preferences for either acetyl-, or butyryl, choline. Both butyrylcholinesterase and AChE exist in two forms in vertebrates (Massoulie and Bon, 1982); globular forms (monomers, dimers, tetramers) and asymetric forms (one two or three tetramers linked to a collagen tail derived from a single precursor protein). In insects, AChE exists predominantly as a globular dimer linked to membrane by a glycophospholipid anchor. In *Drosophila melanogaster* the model proposed contains an active unit of two polypeptides, one 55 kDa and one 18 kDa. Two units are linked together by a disulfide bond at the C terminal end of the 55 kDa polypeptide to form the dimer which is attached to membrane (Fournier *et al.*, 1988; Gnagey *et al.*, 1987). In mosquitoes, studies on *C. pipiens* and *An. albimanus* have indicated a similar model (Bonning, 1989).

Sequence data has been obtained for cDNA coding for the asymetric forms of AChE from *Torpedo californica* (Schumacher *et al.*, 1986) and *T. marmorata* (Sikorav *et al.*, 1987). More recently a cDNA corresponding to the globular dimer form of AChE from *T. marmorata* has been characterised (Sikorav *et al.*, 1988). Both the protein and cDNA sequences have been determined independently for human butyrlcholinesterase (Lockridge *et al.*, 1987; Prody *et al.*, 1987). In insects, the *D. melanogaster* AChE gene has been located and sequenced (Hall and Spierer, 1986).

Molecular Insect Science, Edited by
H. H. Hagedorn *et al.*, Plenum Press, New York, 1990

Altered AChE, associated with relatively low levels of resistance to the OP insecticide, malathion, has been identified in *D. melanogaster* (Morton and Singh, 1982). The gene for this mutant has been cloned, sequenced and compared to the sequence for sensitive AChE. A number of mutations were detected, but all were silent except one, which causes replacement of a phenylalanine with a tyrosine. Comparison with the available data for AChE and butyrylcholinesterase suggested that this particular phenylalanine is highly conserved (Berge and Fournier, 1988; Fournier and Berge, 1990).

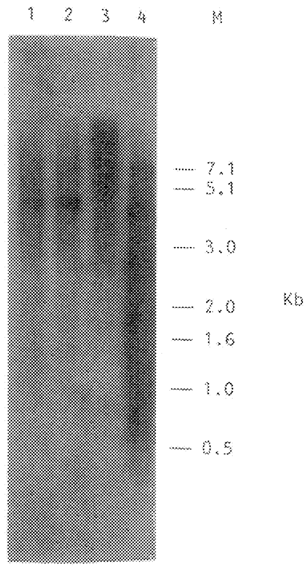


Fig. 1. Hybridisation of the F3/2 cDNA AChE clone (*Pst*I fragment) from *D. melanogaster* to total genomic DNA from *An. stephensi* (1,2), and *An. albimanus* (3) digested with *Hind*III, and *D. melanogaster* (4) digested with *Pst* I.

The importance of AChE as an insecticide target, and its role in a wide spectrum insecticide resistance mechanism, prompted the start of studies to advance knowledge on the structure of the AChE gene in important pest species and to determine the basis of alterations resulting in insecticide resistance. In this context we report here on the cloning, location and initial characterisation of the AChE gene from *An. stephensi*, a major vector of malaria on the Indo-Pakistan sub-continent.

METHODS

Mosquito genomic DNA was prepared by the method of Bingham *et al.* (1981). A genomic DNA library was constructed from the M2 strain (Malcolm and Mali, 1986) of *An. stephensi*. DNA partially digested with *Sau*3A was cloned in the lambda vector EMBL3 following procedures described by Kaiser and Murray (1985). Approximately 180,000 recombinants were obtained.

DNA probes were radiolabelled with α^{32}P-dCTP using random oligonucleotides as primers and Klenow enzyme (Boehringer Mannheim), procedures followed the manufacturers instructions. Details of the DNA sequencing strategy will be reported elsewhere. All other procedures were standard (Maniatis *et al.*, 1982).

In situ hybridisation was performed on polytene chromosomes prepared from ovarian nurse cells. Procedures were essentially the same as those described by Pardue (1986), except that probes were labelled with Biotin-11-dUTP. Biotin detection was performed using a DETEK I-*Hrp* kit (ENZO Biochem, Inc.) following the manufacturers instructions. Chromosome arm designations and numbering are based on the larval salivary gland chromosome map prepared by Sharma *et al.* (1969).

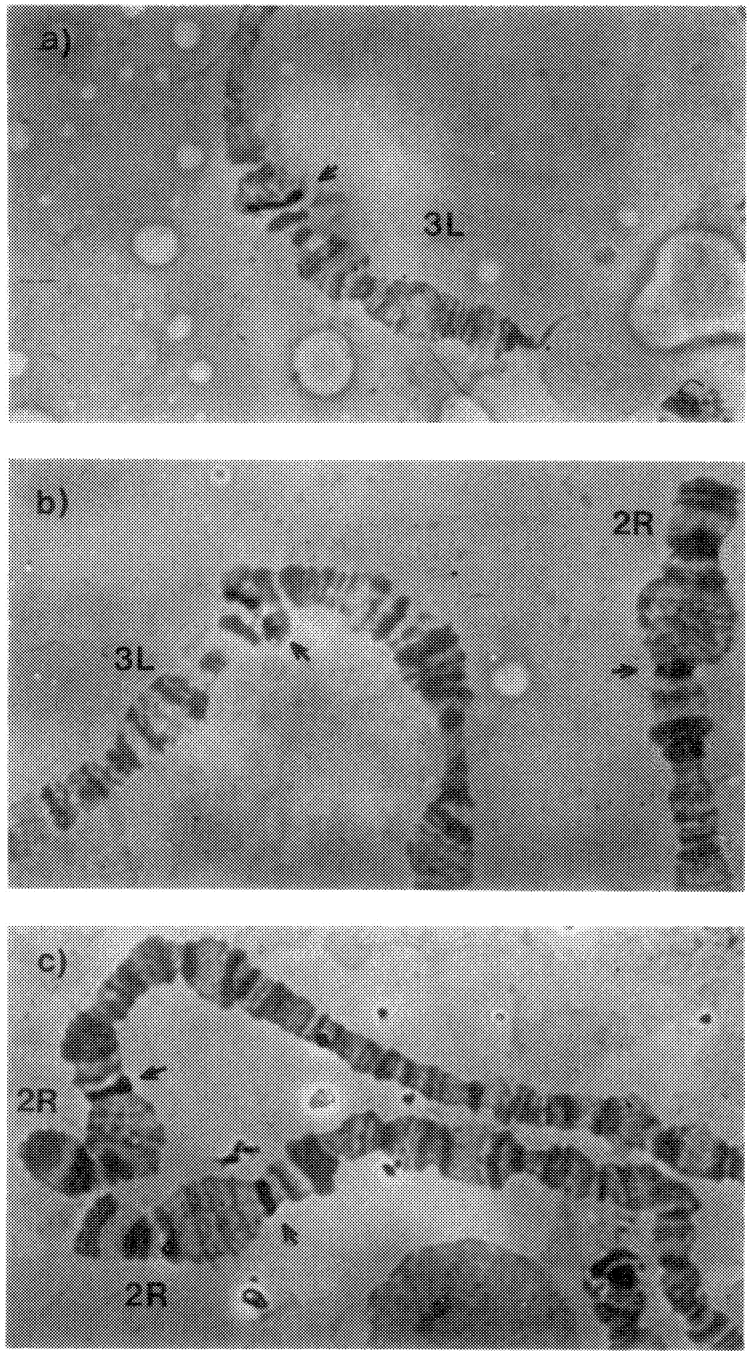


Fig. 2. *In situ* hybridisation of biotin labelled M2-42 to ovarian nurse cell polytene chromosomes. Arrows indicate bands staining positive for biotin on; 2a) chromosome arm 2R; 2b) chromosome arm 3L and 2R; 2c) two chromosome arms 2R.

RESULTS

A *Pst*I cDNA fragment from the F3/2 clone, encompassing most of the coding region of the *D. melanogaster* AChE gene (Hall and Spierer, 1986) was used to probe Southern blots of genomic DNA from a range of mosquito species. Despite varying the hybridisation conditions only DNA from strains of *An. stephensi* produced a positive result. Figure 1. shows an example.

A genomic DNA library from *An. stephensi* was screened unamplified with the *D. melanogaster* AChE probe. Out of three candidate clones obtained, one designated M2-42, was confirmed as positive. Southern blots of restriction digests of M2-42 probed with the *D. melanosgaster* cDNA produced strong hybridisation to a *Pst*I fragment of 1 kb and an *Xho*I fragment of 3.2 kb. Partial sequence determination of the *Pst*I fragment confirmed that it had homology with the *D. melanogaster* AChE gene.

The M2-42 clone was used intact to perform an *in situ* hybridisation to *An. stephensi* polytene chromosomes. Two positive locations were obtained, one a constricted doublet close to the telomere of chromosome arm 2R, at the junction of 7B-C, the other a small puff close to the centromere end of chromosome arm 3L, within 38C (Figures 2a, b and c).

Hybridisation of the *Xho*I fragment from M2-42 to Southern blots of *Xho*I digests of total genomic DNA produced a single positive band of the expected size (3.2 kb) in two strains and the reciprocal F1 progeny of a cross between them. A third strain produced a slightly larger band (3.4kb) as shown in Fig. 3a. The 3.4 kb band could be detected in one of the other strains and the F1s with longer exposure times. The blot was stripped and rehybridised at a lower temperature (37 instead of 42°C). Although more bands could be detected (Figure 3b) these could be accounted for by non-specific hybridisation to repetitive DNA sequences, visible as bands within the DNA smear on the original electrophoresis gels. Probing with the total M2-42 clone under these conditions produced a smear obscuring any additional bands which may have been present.

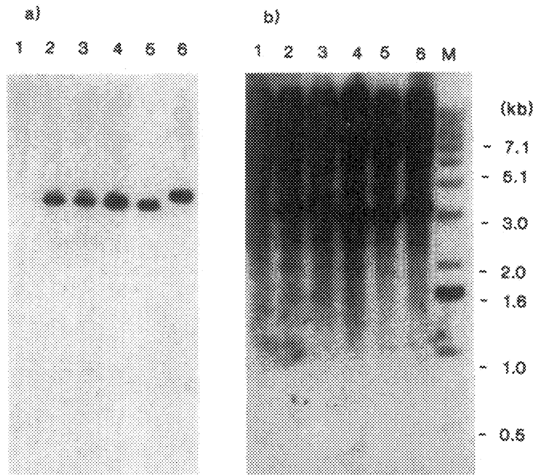


Fig. 3. Hybridisation of the M2-42 clone (XhoI fragment) to *Xho*I digests of total genomic DNA from *D. melanogaster* (1) and *An. stephensi* (2-6). a) 42°C, b) 37°C.

A comparison of the restriction map produced for M2-42 with the one produced by hybridisation of the *Xho*I fragment to Southern blots of total genomic DNA (Figure 4), accounted for all fragments. Using the *D. melanogaster Pst*I cDNA fragment, which comprises DNA coding for amino acids 21 to 535, all of the hybridising sequence in M2-42 was within a 3.2 *Xho*I fragment close to the left arm of the vector. This fragment was completely sequenced and found to contain sequences homologous to all except the last exon of the *D. melanogaster* AChE gene. An adjacent subclone of 550bp provided the

complete sequence of the *An. stephensi gene*. The detailed results of sequencing will be reported in full elsewhere.

The organisation of the *An. stephensi* AChE gene was deduced by comparison to the cDNA sequence of *D. melanogaster*. The gene comprises ten coding exons, coding for 31-99 amino acids, and separated by short introns of 55-105bp. A comparison of the deduced amino acid sequences for the two insect AChEs is shown in Figure 5, excluding the signal peptides which in mosquito was 29 amino acids. The homology overall at the amino acid level was found to be 69%.

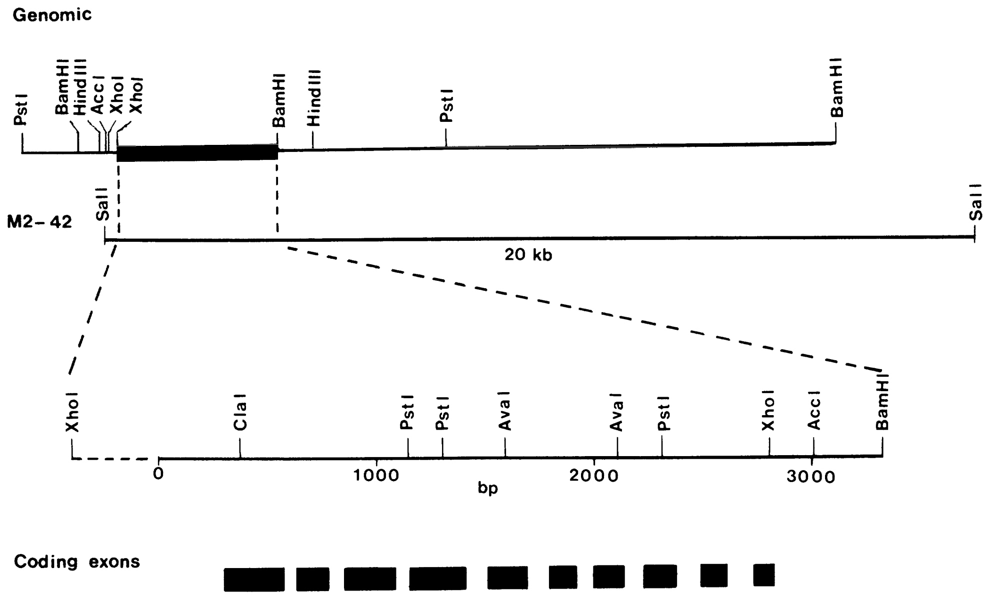


Fig. 4. Genomic organisation of the *An. stephensi* AChE gene.

DISCUSSION

Positive hybridisation results were obtained with the *D. melanogaster* AChE probe to Southern blots of *An. stephensi* total genomic DNA, but were negative for each of the other mosquito species tried, even with lowered stringency. From the results of other projects in the laboratory, it was unlikely that this was due to the quality of DNA, particularly as *An. stephensi* produced consistent results on different blots, even with DNA from different strains. Although the experiments with other species were not exhaustive, it was decided to pursue the *An. stephensi* AChE gene first, rather than persevere with mosquito species in which insensitive AChE has already been found.

In situ hybridisation with the *An. stephensi* AChE clone to polytene chromosomes indicated two locations. This was unexpected, as so far evidence for only one locus in insects has been found, including studies on insensitive AChE (Oppenoorth, 1985). However, in contrast to the chromosome results, Southern blots of total genomic DNA probed with the M2-42 XhoI fragment suggested only one locus, unless the two are identical. The restriction maps obtained (Fig. 4) indicated that the flanking regions would also have to be identical, which seems unlikely. Lowering the stringency of hybridisation with the XhoI fragment produced no evidence that the second locus was a different, but related gene to AChE.

The chromosome location of only one AChE gene has been determined in mosquitoes. This was established from the cytology of a genetic sexing system in *Anopheles albimanus*, which involved a Y translocation tightly linking an insensitive AChE responsible for propoxur resistance. The gene was located towards the telomere of chromosome arm 2R

```
        1       10        20        30        40        50        60        70        80
D.m.  VIDRLVVQTSSGPVRGRSVTVQGREVHVYTGIPYAKPPVEDLRFRKPVPAEPWHGVLDATGLSATCVQERYEYFPGFSGEEIW
      :************:****  ********: *:*:*****: ***:*************** *   *:********** ***:*
A.s.  IIDRLVVQTSSGPIRGRSTMVQGREVHVFNGVPFAKPPVDSLRFKKPVPAEPWHGVLDATRLPPSCIQERYEYFPGFAGEEMW
        1       10        20        30        40        50        60        70        80

            90       100       110       120       130       140       150       160
D.m.  NPNTNVSEDCLYINVWAPAKARLRHGRGANGGEHPNGKQADTDHLIHNGNPQNTTNGLPILIWIYGGGFMTGSATLDIYNADI
      ************:*:* * * ******* * * :       * * : :    :    ** :*:******** *  *******:*
A.s.  NPNTNVSEDCLYLNIWVPTKTRLRHGRGLNFGSNDYFQ..DDDDFQRQ...HQSKGGLAMLVWIYGGGFMSGTSTLDIYNAEI
            90       100       110       120            130       140       150       160

        170       180       190       200       210       220       230       240       250
D.m.  MAAVGNVIVASFQYRVGAFGFLHLAPEMPSEFAEEAPGNVGLWDQALAIRWLKDNAHAFGGNPEWMTLFGESAGSSSVNAQLMS
      :********** ********** *** :    : *:****:*:***********:** ****:*: :******** ***  :*:*
A.s.  LAAVGNVIVASMQYRVGAFGFLYLAPYI.NGYEEDAPGNMGMWDQALAIRWLKENAKAFGGDPDLITLFGESAGGSSVSLHLLS
            170       180        190       200       210       220       230       240

             260       270       280       290       300       310       320       330
D.m.  PVTRGLVKRGMMQSGTMNAPWSHMTSEKAVEIGKALINDCNCNASMLKTNPAHVMSCMRSVDAKTISVQQWNSYSGILSFPSAP
      ****** ***::****:******** ***::*    **:*****  ***  *  ** *** ****************** *****
A.s.  PVTRGLSKRGILQSGTLNAPWSHMTAEKALQIAEGLIDDCNCNLTMLKESPSTVMQCMRNVDAKTISVQQWNSYSGILGFPSAP
         250       260       270       280       290       300       310       320

         340       350       360       370       380       390       400       410
D.m.  TIDGAFLPADPMTLMKTADLKDYDILMGNVRDEGTYFLLYDFIDYFDKDDATALPRDKYLEIMNNIFGKATQAEREAIIFQYTS
      **** *: *****::: *:*    ***:*  ****************:** ** *****:***** ** ** : **********
A.s.  TIDGVFMTADPMTMLREANLEGIDILVGSNRDEGTYFLLYDFIDYFEKDAATSLPRDKFLEIMNTIFNKASEPEREAIIFQYTG
               340       350       360       370       380       390       400       410

               430       440       450       460       470       480       490       500
D.m.  WE.GNPGYQNQQQIGRAVGDHFFTCPTNEYAQALAERGASVHYYYFTHRTSTSLWGEWMGVLHGDEIEYFFGQPLNNSLQYRPV
      ** ** *****:*:********* *****:*  * *******************************:** ****:* *****
A.s.  WESGNDGYQNQHQVGRAVGDHFFICPTNEFALGLTERGASVHYYYFTHRTSTSLWGEWMGVLHGDEVEYIFGQPMNASLQYRQR
           420       430       440       450       460       470       480       490

           510       520       530       540          550       560       570
D.m.  ERELGKRMLSAVIEFAKTGNPAQDGEEWPNFSKEDPVYYIFSTDD....KIEKLARGPLAARCSFWNDYLPKVRSWA....GT
      **:* :**:  * ***:***** :** ** : :*:*:::**  :    . : **  ***:*  * ****:**::* *
A.s.  ERDLSRRMVLSVSEFARTGNPALEGEHWPLYTRENPIFFIFNAEGEDDLRGEKYGRGPMATSCAFWNDFLPRLRAWSVPSKSP
       500       510       520       530       540       550       560       570

        580       590       600                      610
D.m.  CD..GDSGSASISPRLQLLGIAALIYI..CA...ALRTK............RVF
      *:   :   **:*  : :: :  *: *  **   *::              * *
A.s.  CNLLEQMSIASVSSTMPIVVMVVLVLIPLCAWWWAIKKNKTPPHPQVILETRAFMH
                590       600       610       620       630  635
```

Fig. 5. Comparison of deduced *An. stephensi (A.s.)* and *D. melanogaster (D.m.)* AChE protein sequences. Sequence alignment programme by Corpet (1988), using Dayhoff replacement values.

(Kaiser *et al.*, 1979),therefore apparently similar to position 7B-C obtained in *An. stephensi*. It has been argued that there is evidence to generalise linkage group chromosome correlations, established for one species of anopheline to any other (Hunt, 1987). Data of this sort are not abundant in mosquitoes, but there are perhaps sufficient data for *An. stephensi* and *An. albimanus* to support the hypothesis with respect to these two species, even although they belong to different subgenera. This would therefore predict that the chromosome 2 location in *An. stephensi* is more likely to be correct.

The 38C location on chromosome arm 3L is interesting, because it has been previously described within the context of a comparative study of homologous banding patterns between polytene chromosomes from the larval salivary glands and ovarian nurse cells. In salivary gland chromosomes no puff equivalent was found within 38C, whereas in ovarian nurse cells this region was found to incorporate ^{3}H-uridine relatively intensely, confirming its classification as a puff. This suggested a difference in gene expression within this region between the germline and somatic polytene chromosomes, in contrast to the overall findings which indicated very similar patterns of gene expression (Redfern, 1981). The observed restriction fragment length polymorphism with Xho I digests (Figure 3), provides a useful marker for linkage studies and these are currently being undertaken to resolve the location of AChE.

The *An. stephensi* AChE gene contains 10 coding exons within 3 kb of genomic DNA, in contrast to *D. melanogaster* where the coding exons were spread over 20 kb. The position of introns in the mosquito genome was identical to those recently determined in *D. melanogaster* (Fournier *et al.*, 1989), except the equivalent of exon 4 was split by an additional intron in the mosquito.

The deduced protein sequence (Figure 5) showed a relatively high degree of homology with the *D. melanogaster* AChE gene. One of the regions most widely diverged between the two proteins, between residues 112 and 134, is hydrophilic in both insect sequences and probably contains the site, or sites, at which the enzyme undergoes proteolytic cleavage. This would be consistant with the biochemical data for mosquito AChE, which indicated a similar model for the molecular form of the enzyme to that proposed in *Drosophila* (Bonning 1989, Gnagey *et al.*, 1987; Fournier *et al.*, 1988). Vertebrate cholinesterases, which do not undergo cleavage, this region has no equivalent in the sequences available (Schumacher *et al.*, 1986; Sikorav *et al.*, 1987; 1988; Lockridge *et al.*, 1987; Prody *et al.*, 1987).

The active site contains a serine located at position 238 (Fig. 5) within a block of six conserved amino acids found in all cholinesterase sequences so far available. Similarly two conserved histidine residues (at positions 460 and 475) are also present in mosquito AChE, one of which may be involved in the catalytic site. The location of the anionic site, implicated in the binding of quaternary ammonium, has been determined from photoaffinity labelling studies on electric eel AChE (Kieffer *et al.*, 1986). At the equivalent location in the insect AChEs (368-371), the sequence is conserved between the two species, but is different from the anionic site sequence of vertebrate AChE, which in turn is different from human butyrlcholinesterase. This may be expected as the three classes of enzyme differ in their substrate affinities.

The phenylalanine residue substituted for tyrosine in the insecticide insensitive mutant of AChE in *D. melanogaster* (Berge and Fournier, 1988; Fournier and Berge, 1989) is located at position 330 in Figure 5. A phenylalanine is also predicted at this position in the mosquito AChE, and most of the flanking blocks are also conserved between the two insects, providing further evidence that this residue is highly conserved and may be important in determining sensitivity to insecticide inhibition.

Further studies based on the available AChE sequence data are now underway to clone and characterise the insecticide sensitive and insensitive AChE genes from the mosquito.

AKNOWLEDGMENTS

Many thanks to Rebecca Reiss (Cornell University), for her help with the *in situ* hybridisation procedures, to Gina Banks for frequent assistance, and to M. Miles and members of the Wolfson Unit (LSHTM) for helpful advice. This work was supported by the Wellcome Trust.

REFERENCES

Berge, J.B. and Fournier, D. 1988. Advances in molecular genetics of acetylcholinesterase insensitivity in insecticide resistant insects. Proc. XVIII Int. Cong. Entomol. Vancouver, B.C. Canada, p 461.

Bingham, P.M., Lewis, R. and Rubin, G.M. 1981. Cloning of DNA sequences from the white locus of *Drosophila melanogaster* by a novel and general method. Cell 25: 693-704.

Bonning, B.C. 1989. "Acetylcholinesterase and insecticide resistance in mosquitoes." Ph.D. Thesis, University of London.

Corpet, F. 1988. Multiple sequence alignment with hierarchical clustering. Nuc. Acids Res. 16: 10881-10890.

Fournier, D. and Berge, J.B. 1990. Resistance to insecticide: Detection of a mutation in the acetylcholinesterase gene from *Drosophila melanogaster*. pp in: "Molecular Insect Science." Hagedorn, H.H., Hildebrand, J.G., Kidwell, M.G. and Law, J.H., eds. Pergamon Press.

Fournier, D., Bride, J.M., Karch, F. Berge, J.B. 1988b. Acetylcholinesterase from *Drosophila melanogaster*: identification of two subunits encoded by the same gene. FEBS Lett. 238: 333-337.

Fournier, D., Karch, F., Bride, J.M., Hall, L.M.C., Berge, J.B. and Spierer P. 1989. The *Drosophila melanogaster* acetylcholinesterase gene: structure, evolution and mutations. J.Mol. Biol. 210: 15-22.

ffrench-Constant, R.H. and Bonning, B.C. 1989. Rapid microtitre plate test distinguishes insecticide resistant acetylcholinesterase genotypes in the mosquitoes *Anopheles albimanus, An. nigerrimus* and *Culex pipiens*. Med. Vet. Entomol. 3: 9-16.

Gnagey, A.L., Forte, M. and Rosenberry, T.L. 1987. Isolation and characterisation of acetylcholinesterase from *Drosophila*. J.Biol.Chem. 262: 13290-13298.

Hall, L.M.C. and Spierer, P. 1986. The *Ace* locus of *Drosophila melanogaster*: structural gene for acetylcholinesterase with an unusual 5' leader. EMBO Journal. 5: 2949-2954.

Hunt, R.H. 1987. Location of genes on chromosome arms in the *Anopheles gambiae* group of species and their correlation to linkage data for anopheline mosquitoes. Med.Vet. Entomol. 1: 81-88.

Kaiser, K. and Murray, N. 1985. The use of phage lambda replacement vectors in the construction of representative genomic DNA libraries. pp. 1-47 in: "DNA Cloning." Vol I. Glover, D.M.ed. IRL Press, Oxford.

Kaiser, P.E., Seawright, J.A. and Joslyn, D.J. 1979. Cytology of a genetic sexing system in *Anopheles albimanus* Wiedmann. Can. J. Genet. Cytol. 21: 201-211.

Kieffer, B., Goeldner, M., Hirth, C., Aebersold, R. and Chang, J.Y. 1986. Sequence determination of a peptide fragment from electric eel acetylcholinesterase involved in the binding of quaternary ammonium. FEBS Lett. 202: 91-96.

Lockridge , O., Bartels, C.F., Vaughan, T.A., Wong, C.K. Norton, S.E. and Johnson, L.L. 1987. Complete amino acid sequence of human serum cholinesterase. J.Biol.Chem. 262: 549-557.

Malcolm, C.A. and Mali, P. 1986. Genetic sexing of *Anopheles stephensi* with the larval morphological mutant *Bl*. Genetica 70: 37-42.

Maniatis, T. Fritsch, E.F., Sambrook, J. 1982. "Molecular Cloning. A Laboratory Manual." Cold Spring Harbor Laboratory, New York.

Massoulie, J. and Bon, S. 1982. The molecular forms of cholinesterase and acetyl - cholinesterase in vertebrates. Annu. Rev. Neurosci. 5: 57-106.

Morton, R.A. and Singh, R.S. 1982. The association between malathion resistance and acetylcholinesterase in*Drosophila melanogaster*. Biochem. Genet. 20: 179-198.

O'Brian, R.D. 1976. Acetylcholinesterase and its inhibition. pp. 271-296 in: "Insecticide biochemistry and physiology" Wilkinson, C.F., ed., Heyden Press.

Oppenoorth, F.J. 1985. Biochemistry and genetics of insecticide resistance. pp. 735-745 in: "Comprehensive Insect Physiology, Biochemistry and Pharmacology." vol 12. G.A. Kerkut and L.I. Gilbert, eds. Pergamon Press, Oxford.

Pardue, M.L. 1986. *In situ* hybridisation to DNA of chromosomes and nuclei. pp. 111-137 in: "Drosophila a Practical Approach." Roberts, D.B. ed. IRL Press Oxford.

Prody, C.A., Zevin-Sonkin, D., Gnatt, A. Goldberg, O. and Soreq, H. 1987. Isolation and characterisation of full length cDNA clones coding for cholinesterase from fetal human tissues. Proc. Natl. Acad. Sci. USA 84: 3555-3559.

Redfern, C.F. 1981. Homologous banding patterns in the polytene chromosomes from larval salivary glands and ovarian nurse cells of *Anopheles stephensi*. Chromosoma (Berl.) 83: 221-240.

Schumacher, M., Camp, S., Maulet, Y., Newton, M., Macphee-Quigley, K., Taylor, S.S., Friedmann, T. and Taylor, P. 1986. Primary structure of *Torpedo californica* acetylcholinesterase deduced from its cDNA sequence. Nature 319: 407-409.

Sharma, G.P., Parshad, R., Narang, S.L. and Kitzmiller, J.B. 1969. The salivary chromosomes of *Anopheles stephensi*. J. Med. Ent. 6: 68-71.

Sikorav, J.L., Krejci, E. and Massoulie, J. 1987. cDNA sequences of *Torpedo marmorata* acetylcholinesterase: primary structure of the precursor of a catalytic subunit; existance of multiple 5'-untranslated regions. EMBO Journal 6: 1865-1873.

Sikorav, J.L., Duval, N., Anselmet, A., Bon, S., Krejci, E., Legay, C., Osterlund, M., Reimund, B. and Massoulie, J. 1988. Complex alternative splicing of acetylcholinesterase transcripts in *Torpedo* electric organ; primary structure of the precursor of the glycolipid-anchored dimeric form. EMBO Journal 7: 2983-2993.

THE CLONING AND EXPRESSION IN *Xenopus laevis* OOCYTES OF AN INSECT NICOTINIC ACETYLCHOLINE RECEPTOR α-SUBUNIT

John Marshall, Eric A. Barnard[1] and David B. Sattelle[2]

Department of Pharmacology, Yale University, 333 Cedar Street, New Haven, CT 06510, U.S.A.

[1]MRC Molecular Neurobiology Unit, Hills Road, Cambridge CB2 2QH, U.K.
[2]AFRC Unit of Insect Neurophysiology and Pharmacology, Downing Street, Cambridge, CB2 3EJ, U.K.

INTRODUCTION

The central nervous system of an insect shares many chemical and some organizational features evident in the more complex nervous systems of vertebrates. In both groups of organisms nerve cells form networks that control behaviour. The functions of an increasing number of uniquely identifiable neurones are established in events such as flight and respiration of insects (Hoyle and Burrows, 1973a,b; Robertson, 1986). In order to understand fully these behaviours it is important to study the molecular components of the system. Of particular interest are the synapses which are control points in the communication between neurones. The chemical synapse plays a major role in neural integration and many different types of neurotransmitter molecules have been recognized such as acetylcholine (ACh), γ-aminobutyric acid (GABA), octopamine and glutamic acid which in turn activate their respective receptors. Although many of these neurotransmitters are also present in the vertebrate CNS, it is becoming increasingly clear that some insect receptors including the nicotinic acetylcholine receptor (nAChR) have different pharmacological properties from those of their vertebrate counterparts (Sattelle, 1980, 1988; Benson, 1988).

The nAChR subunits identified in vertebrate muscle and nervous tissue are compared in Fig. 1. The vertebrate muscle nAChR is a pentameric glycoprotein composed of four different subunits with a stoichiometry of $\alpha 2\beta$ (γ or ε) δ (Raftery et al., 1980), whereas the CNS nAChRs are made up of only an α and a non-α subunit (Whiting and Lindstrom, 1987). All the subunits of the muscle, three different neuronal α subunits (α2, α3, α4), and two neuronal non-α subunits (β2 and β3) have been cloned (Numa, 1986; Goldman et al., 1987; Nef et al., 1988). Expression of these cDNAs using the *Xenopus laevis* oocyte system demonstrates that all four subunits of the muscle and two neuronal subunits (an α subunit e.g. α2, α3, α4 in combination with the β2 subunit) are required for the formation of fully functional nAChRs (Boulter et al., 1987; Deneris et al., 1988).

The muscle nAChRs are specifically antagonized by the snake (*Bungarus multicinctus*) polypeptide toxin, α-bungarotoxin (α-Bgt). A distinct but closely related polypeptide κ-bungarotoxin (κ-Bgt) blocks all neuronal nAChRs tested to date whereas only in a few cases does α-Bgt antagonize nicotinic cholinergic transmission in the nervous system (Freeman et al., 1980; de la Garza et al., 1987).

In insects two different putative nAChR cDNAs have been isolated from *Drosophila melanogaster*, a non-α subunit (ARD) and an α-like subunit (ALS). It remains to be established whether or not they are constituents of a functional nAChR (Hermans-Borg-meyer et al., 1986; Bossy et al., 1988). In the locust, *Locusta migratoria* (Breer et al., 1985)

Molecular Insect Science, Edited by
H. H. Hagedorn *et al.*, Plenum Press, New York, 1990

and the cockroach, *Periplaneta americana* (Sattelle and Breer, 1985), a protein with M_r = 65,000 on denaturing polyacrylamide gels has been purified by α-Bgt affinity chromatography and this protein can be reconstituted into planer lipid bilayers to form an ACh-operated cation channel (Hanke and Breer, 1986).

In the present chapter we describe the molecular characterization of a putative neuronal α-subunit cDNA from the locust, *Schistocerca gregaria.* In addition we show that this α-subunit, named αL1 (also known as ARL2, cf. Marshall et al., 1988), can be expressed in *X. laevis* oocytes to form a functional nAChR with many of the pharmacological properties of the *in vivo* insect nAChR. Therefore, although the amino acid sequence of the locust receptor is homologous to the vertebrate nAChRs, its pharmacological properties are substantially different.

NICOTINIC ACETYLCHOLINE RECEPTORS	SUBUNITS
Vertebrate muscle Torpedo electroplax }	$\alpha_1 \beta_1 \gamma \delta (\epsilon)$
Vertebrate CNS	$\alpha_2 \beta_2$ $\alpha_3 \beta_3$ α_4 α_5
Ancestral molecule	homo-oligomer

Fig. 1. Nicotinic acetylcholine receptor (nAChR) subunits identified in vertebrate muscle and nervous tissue are shown.

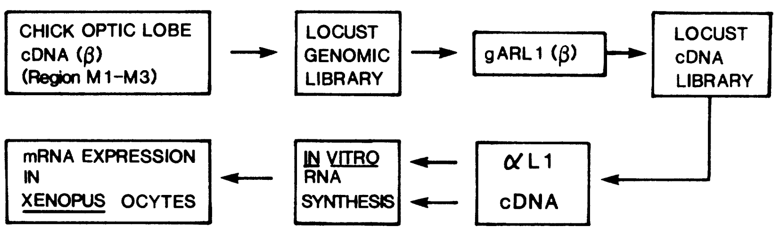


Fig. 2. Schematic outline of the experimental approach adopted in isolating a genomic clone for a putative locust nAChR α-subunit.

ISOLATION OF A GENOMIC CLONE FOR A PUTATIVE LOCUST nAChR SUBUNIT

An outline of the approach used in isolating a genomic clone for a putative locust nAChR is illustrated in Fig. 2.

A locust genomic library constructed in the bacteriophage vector 2001 has been screened with a 308 bp cDNA fragment encoding the transmembrane domains Ml to M3 of the chicken neuronal β2 cDNA under reduced stringency (Marshall et al., 1988a, b). Out of 200,000 recombinants only one clone, gALl, has been isolated. An approximately 400 bp *BamHl-Sacl* restriction fragment of this clone has been sequenced and is found to be homologous to transmembrane domains Ml to M3 and part of the extracellular region including the two cysteines that correspond to cys 128 and cys 142 found in the *Torpedo* α-subunit. The genomic clone does not possess the two adjacent cysteines, cys 192 and cys 193 (*Torpedo* amino acid numbering) present in all α-subunits. Therefore this genomic clone is considered to encode a non-α type of subunit.

```
LOCUST ALPHA   -23 MAAAL  PPMLLLLLLLLLLHHPAAANPDAKRLYDDLLSNYNRLIRPVSNN
DROSOPHILA         MSVL     FAAVFIALHFATGGLANPDAKRLYDDLLSNYNRLIRPVGNN
RAT ALPHA3         MGSVL    FAAVFIALHFATGGLANPDAKRLYDDLLSNYNRLIRPVGNN
CHICK MUSCLE       MEICR      VLLLIFSAAGPGLCYEHETRLVDDLFREYSKVVRPVENH
                   *                             ** ***   *    *** **

LOCUST ALPHA    26 TDTVLVKLGLRLSQLIDLNLKDQILTTNVWLEHEWQDHKFRWDPAEYGGV
DROSOPHILA         SDRLTVKMGLRLSQLIDVNLKNQIMTTNVWVEQEWNDYKLKWNPDDYGGV
RAT ALPHA3         SHPVIIQFEVSMSQLVKVDEVNQIMETNLWLKQIWNDYKLKWKPSDYQGV
CHICK MUSCLE       RDAVVVTVGLQLIQLINVDEVNQIVTTNVRLKQQWTDINLKWNPDDYGGV
                                ** 	      **  **      * *    ***  * **

LOCUST ALPHA    76 TELYVPSEHIWLPDIVLYNNADGEYVVTTMTKAVLHHTGKVVWTPPAIFK
DROSOPHILA         DTLHVPSEHIWHPDIVLYNNADGNYEVTIMTKAILHHTGKVVWKPPAIYK
RAT ALPHA3         EFMRVPAEKIWKPDIVLYNNADGDFQVDDKTKALLKYTGEVTWIPPAIFK
CHICK MUSCLE       KQIRIPSDDIWRPDLVLYNNADGDFAIVKYTKVLLEHTGKITWTPPAIFK
                        *   ** ** ********       **  *  **   * ******

LOCUST ALPHA   126 SSCEIDVRYFPFDQQTCFMKFGSWTYDGDQIDLKHINQKYDDNKVKVGID
DROSOPHILA         SFCEIDVEYFPFDEQTCFMKFGSWTYDGYMVDLRHLKQTADSDNIEVGID
RAT ALPHA3         SSCKIDVTYFPFDYQNCTMKFGSWSYDKAKIDLVL          IGSSMN
CHICK MUSCLE       SYCEIIVTYFPFDQQNCSMKLGTWTYDGTMVVINP          ESDRPD
                   * * * * ***** * * ** * * ***   *

LOCUST ALPHA   176 LREYYPSVEWDILGVPAERHEKYYPCCAE PYPDIFFNITLRRKTLFYTV
DROSOPHILA         LQDYYISVEWDIMRVPAVRNEKFYSCCEE PYLDIVFNLTLRRKTLFYTV
RAT ALPHA3         LKDYWESGEWAIIKAPGYKHEIKYNCCEE IYQDITYSLYIRRLPLFYTI
CHICK MUSCLE       LSNFMESGEWVMKDYRGWKHWVYYACCPDTPYLDITYHFLMQRLPLYFIV
                   *     * **             * **     * **       *  *
                                                               ------
LOCUST ALPHA   225 NLIVPCVGISYLSVLVFYLPADSGEKIALCISILLSQTMFFLLISEIIPS
DROSOPHILA         NLIIPCVGISFLSVLVFYLPSDSGEKISLCISILLSLTVFFLLLAEIIPP
RAT ALPHA3         NLIIPCLLISFLTVLVFYLPSDCGEKVTLCISVLLSLTVFLLVITETIPS
CHICK MUSCLE       NVIIPCLLFSFLTGFVFYLPTDSGEKMTLSISVLLSLTVFLLVIVELIPS
                   * * **   * *   ***** * ***  * ** ******* *   * **
                   -------M1----------      ---------M2---------
LOCUST ALPHA   275 TSLALPLLGKYLLFTMVLVGLSVVITIMVLNVHYRKPSTHKMAPWVRKVF
DROSOPHILA         TSLTVPLLGKYLLFTMMLVTLSVVVTIAVLNVNFRSPVTHRMAPWVQRLF
RAT ALPHA3         TSLVIPLIGEYLLFTMIFVTLSIVITVFVLNVHYRTPTTHTMPTWVKAVF
CHICK MUSCLE       TSSAVPLIGKYMLFTMVFVIASIIITVIVINTHHRSPSTHTMPPWVRKIF
                   **   ** * * ****  *  *   *  * *   * * ** *  **   *
                             ----------M3----------
LOCUST ALPHA   325 IRRLPKLL LMRVPEQLLADLASKRLLRHAHNSKLSAAAAAAVAAAASSS
DROSOPHILA         IQILPNLLCIERPKKEEPEEDQPPEVLTDVYHLPPDVDKFVNYDSKRFSG
RAT ALPHA3         LNLLPRVMFMTRPTSGEGDTPKTRTFYGAELS   NLNCFSRADSKSCKE
CHICK MUSCLE       IDTIPNIMFFSTMKRPSRDKPDKKIFAEDI     DISEISGKQGPVPVN
                       *

LOCUST ALPHA   374 AASSPDSLRHHHLHQHQHQHHLQLHHLQRPG  GCNGLHSATNRFGGSAG
DROSOPHILA         DYGIP ALPASHRFDLAAAGGISAHCFAEPPLPSSLPLPGADDDLFSPSG
RAT ALPHA3         GYPCQDGTCGYCHHRRVKISNFSAN LTRSS                SSES
CHICK MUSCLE       FYSP                      LTKNP

LOCUST ALPHA   422 AFGGLPSV   VGLDGSLSDVATR  KKYPFELEKAIHNVLFIQNHMQRQ
DROSOPHILA         LNGDISPGCCPAAAAAAAADLSPTFEKPYAREMEKTIEGSRFIAQHVKNK
RAT ALPHA3         VNAVLS            LSALSP     EIKEAIQSVKYIAENMKAQ
CHICK MUSCLE                                    DVKNAIEGIKYIAETMKSD
                                                     *     *
                                                -------MA--------
LOCUST ALPHA   467 DEFDAEDQDWGFVAMVLDRLFLWIFTIASIVGTFAILCEAPALYDDTKPI
DROSOPHILA         DKFESVEEDWKYIAMVLDRMFLWIFAIACVVGTAMIILQAPSLHDQSQPI
RAT ALPHA3         NVAKEIQDDWKYVAMVIDRIFLWVFILVCILGTAGLFLQPLMARDDT
CHICK MUSCLE       QESSNAADEWKFVAMVLDHLLLVIFMLVCIIGTLAVKAGRLIELNQQG
                            *   *** *   *  *      **
                                   ---------M4---------
LOCUST ALPHA   517 DMELSSVAQQQF LPDIDF
DROSOPHILA         DILYSKIAKKKFELLKMGSENTL
```

Fig. 3. Sequence alignment of the locust (*Schistocerca gregaria*) nicotinic acetylcholine receptor subunit αL1 (=LOCUST ALPHA) with neuronal and muscle sequences of vertebrates and a *Drosophila melanogaster* sequence (ALS).

ISOLATION OF THE cDNA ENCODING A LOCUST nAChR SUBUNIT

The 400 bp *BamHl-Sacl* restriction fragment of gARLl has been used to screen a locust embryonic cDNA library constructed in the bacteriophage vector λgt10. A weakly hybridizing clone which also hybridizes strongly to a 'best guess' 45-base oligonucleotide corresponding to amino acids 132 to 146 of muscle nAChR subunits, has been isolated and sequenced (Marshall et al., unpublished results).

Comparison of the locust amino acid sequence with vertebrate sequences is shown in Fig. 3 and reveals striking homology. The locust subunits are more conserved with the vertebrate neuronal nAChR subunits than muscle nAChR subunits. Thus in the transmembrane region M1 - M3, which is the most conserved domain, the locust α-subunit is 73% identical to the chick brain α3-subunit and 64% identical to the chick muscle α-subunit.

THE LOCUST nAChR IS A MEMBER OF A LIGAND-OPERATED ION CHANNEL SUPERFAMILY

The locust polypeptide possesses all of the characteristic features of neurotransmitter receptor subunits (Barnard et al., 1989). These include four hydrophobic putative membrane-spanning domains, two cysteine residues at positions 128 and 142 that are known to form a disulphide bond in the *Torpedo* nAChR (Kao and Karlin, 1986). This cysteine-loop region is also conserved in the $GABA_A$ and glycine receptor (Barnard et al., 1989). In addition a pair of adjacent cysteine residues at positions 201 and 202 which are common to all α-subunit types may be close to the ACh binding site (Kao et al., 1984) and have been implicated in the binding of α-Bgt. The presence of this cysteine pair defines this subunit as an α-type.

The loop between M3 and M4 is non-conserved although an amphipathic sequence, a strong feature of all nAChRs, is found adjacent to the M4 domain. This amphipathic helix is not present in the anion channel subunits of the $GABA_A$ and glycine receptor which suggests that this helix may be particularly important in the function of ligand-operated cationic channels.

A diagram summarizing the topology of the αL1-subunit is shown in Fig. 4. A similar topology has been predicted for vertebrate nAChR, $GABA_A$ and glycine receptors which have revealed the existence of a ligand-gated cation and anion channel superfamily (Schofield et al., 1986; Grenningloh et al., 1987). This superfamily is deduced to have a common evolutionary history and so insect receptors may represent a more primitive form of the vertebrate receptors.

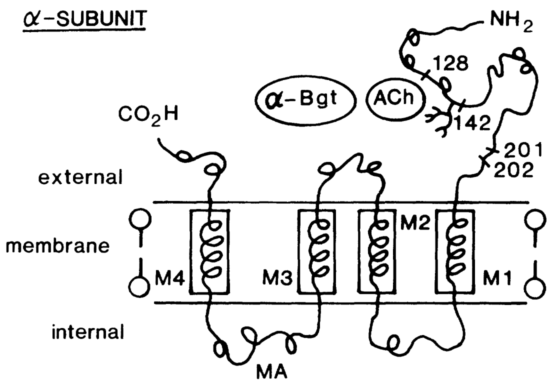


Fig.4. Schematic representation of the topology of the locust αL1 nicotinic acetylcholine receptor (nAChR) subunit. The precise sites of binding of acetylcholine (ACh) and α-bungarotoxin (α-Bgt) remain to be determined.

FUNCTIONAL EXPRESSION OF THE LOCUST α-SUBUNIT

Using the cloned αL1 cDNA, RNA is prepared by *in vitro* transcription, polyadenylated using *E. coli* Poly A polymerase and microinjected into *X. laevis* oocytes (Marshall et al., unpublished results). The pharmacology of the response of the expressed receptors to nicotine has been determined. At concentrations of 1.0 x 10^{-6}M, both α-Bgt and κ-Bgt completely block the nicotine-induced response (Fig. 5). Unlike the vertebrate nAChRs reported to date, the *in vivo* insect nAChR is inhibited by both α-Bgt and κ-Bgt (Sattelle et al., 1983; Pinnock et al., 1988; Chiappinelli et al., 1989).

Strychnine, a vertebrate glycine receptor antagonist is effective at inhibiting the expressed and the *in vivo* insect receptor (Benson, 1988a). In addition bicuculline, a vertebrate $GABA_A$ receptor antagonist which fails to antagonize insect $GABA_A$ (Wafford et al., 1987; Sattelle et al., 1988) is able reversibly to antagonize the responses to nicotine of the cloned locust nAChR expressed in the oocyte membrane. *In vivo* bicuculline antagonizes responses to ACh in the locust (Benson, 1988b) and the cockroach (Buckingham et al., 1989). The pharmacological studies on the expression of the αL1-subunit are summarized in Table 1 and are compared with those of the vertebrate nAChR and an *in vivo* insect nAChR.

Table 1. Pharmacology of nAChR Subtypes

LIGAND	VERTEBRATE		INSECT			
	Muscle	Nervous system	Cloned Locust	Locust somata[1]	Cockroach somata[2,3,4]	Cockroach synaptic[5,6,7]
Nicotine	agonist	agonist	agonist	agonist	agonist	agonist
Tetraethyl-ammonium	(K^+ channel blocker)		blocker	blocker	N.T.	blocker
α-Bungarotoxin	blocker		blocker	blocker	blocker	blocker
κ-Bungarotoxin		blocker	blocker	blocker	blocker	blocker
D-Tubo curarine	blocker	blocker	blocker	blocker	blocker	blocker
Mecamylamine	weak blocker	blocker	blocker	blocker	blocker	blocker
Bicuculline		$GABA_A$ antagonist	blocker	blocker	blocker	blocker
Strychine		Glycine antagonist	N.T.	blocker	N.T.	N.T.
Picrotoxin		GABA/Cl^- channel blocker	N.T.	blocker	blocker	blocker

1. Benson, J.A. 1988.
2. David, J.A. and Sattelle, D.B. 1984.
3. Walker, R.J., Crossman, A.R., Woodruff, G.N., and Kerkut, G.A. 1971.
4. Pinnock, R.D., Lummis, S.C.R., Chiappinelli, V.A., and Sattelle, D.B. 1988.
5. Sattelle, D.B., Harrow, I.D., Hue, B., Pelhate, M., Gepner, J.I., and Hall, L.M. 1983.
6. Chiappinelli, V.A., Hue, B., Mony, L., and Sattelle, D.B. 1989.
7. Sattelle, D.B. 1978.

Synaptic, α-Bgt-sensitive, neuronal nAChRs are present on insect interneurones (Sattelle et al., 1980; Sattelle et al., 1983) and motor neurones (Carr and Fourtner, 1980). Non-synaptic (cell body) nAChRs that are also sensitive to αBgt are also present on the cell body membrane of the interneurone GI2 (Harrow and Sattelle, 1982) and motor neurone D_f (David and Sattelle, 1984) of the cockroach.

For the non-synaptic receptor on motor neurone D_f detailed agonist and antagonist pharmacological profiles have been constructed (Sattelle and David, 1983; David and Sattelle, 1984). Voltage-clamp experiments indicate that this α-Bgt-sensitive receptor gates a cation channel permeable to Na^+, K^+ and Ca^{2+}.

In the cockroach metathoracic ganglion, which contains motor neurone D_f, dorsal unpaired median (DUM) neurones can be located and AChRs on the cell body membrane of these modulator cells respond to nicotine but appear to be much less sensitive to α-Bgt than the counterpart cell body receptors on motor neurone D_f (Lane et al., 1982) and the A6 ganglion interneurone GI2 (Harrow and Sattelle, 1982). Since the cell body membrane of DUM cells is depolarized by nicotine, it may be that the receptors on this cell represent a subtype of nAChR with reduced α-Bgt sensitivity. Unidentified cultured embryonic neurones that respond to nicotine but are insensitive to α-Bgt, though very much in the minority compared to α-Bgt-sensitive receptors, do exist (Lees et al., 1983). This provides pharmacological evidence that distinct subtypes of neuronal nAChRs are present in embryonic as well as adult insects. Molecular evidence for an α-Bgt-insensitive nAChR has been obtained by Bossy et al., (1988). These authors have reported that a β-galactosidase fusion protein of the *D. melanogaster* α-like subunit (ALS) does not bind α-Bgt, suggesting that this gene encodes an α-Bgt-insensitive receptor.

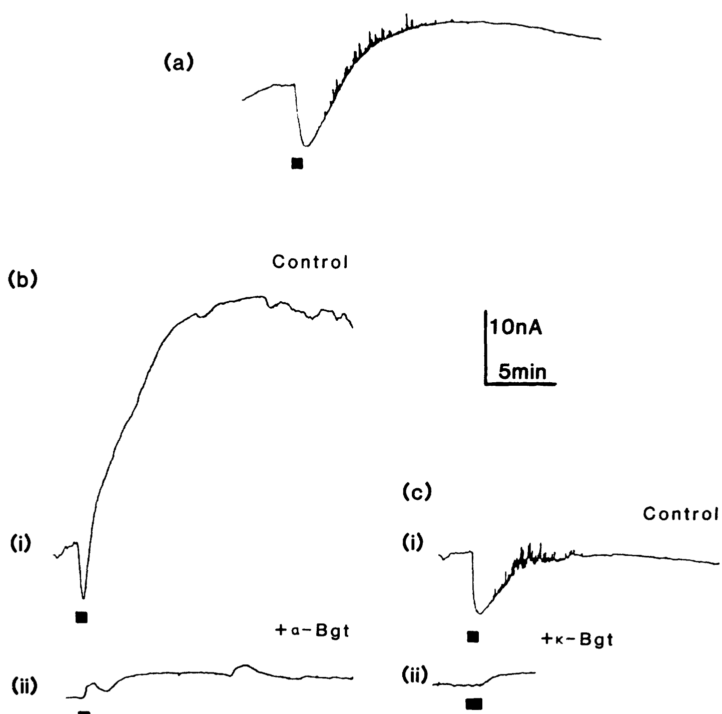


Fig. 5. Nicotine-induced currents recorded from *Xenopus laevis* oocytes injected with *in vitro* transcribed locust RNA. (a) The response to bath-applied nicotine is a transient inward current. In some cells this is followed by a slower outward current. (b) Nicotine-induced current is blocked by 1.0×10^{-6} M α-bungarotoxin (α-Bgt). (c) Nicotine-induced current is blocked by 1.0×10^{-7} M κ-bungarotoxin. As is the case for the in vivo receptor, the expressed locust nAChR is blocked by both α-bungarotoxin and κ-bungarotoxin.

CONCLUSIONS

Expression of a single cloned locust α-like cDNA in the *X. laevis* oocyte is sufficient for the formation of a functional ion channel with the characteristic pharmacology of an insect neuronal nAChR. It seems likely that this single subunit is capable of self-assembly to form a homo-oligomeric structure which, if compared to the *Torpedo* nAchR, would be pentameric. However a tetrameric structure cannot yet be ruled out, though such a state would need a re-evaluation of current concepts of subunit interactions. Also we cannot rule out a hetero-oligomeric complex incorporating an intrinsic component of the oocyte.

It has already been reported by Breer et al. (1985) that the locust α-Bgt binding protein has a molecular size in the range M_r = 250,000-300,000 on non-denaturing polyacrylamide gels. Upon denaturation, this material yields a single band of M_r = 65,000. The fact that this purified receptor can be reconstituted to form functional channels in planar lipid bilayers (Hanke and Breer, 1986) suggests that the αL1-subunit may encode the locust M_r 65,000 component. Both the reconstitution experiments and the *X. laevis* expression studies support the notion that under these experimental conditions a homo-oligomeric form of the nAChR can be created.

The formation of functional ligand-gated ion channels upon expression of a single subunit in *Xenopus* oocytes has also been observed for the vertebrate $GABA_A$ receptor (Blair et al., 1988) and the glycine receptor (Schmieden et al., 1989), both of which have hetero-oligomeric structures (reviewed in Stephenson, 1988). The pharmacological properties of these presumed homo-oligomeric artefacts closely resemble the *in vivo* pharmacology. Therefore, although the pharmacology of the express αL1-subunit matches all aspects of the *in vivo* pharmacology so far tested, it remains to be seen whether or not an structure is found *in vivo*. In addition we have also obtained a locust non-α subunit which may be the homologue of the *D. melanogaster* ARD non-α subunit since they share 89% identity at the amino acid level (Hermans-Borgmeyer et al., 1986). The ARD subunit is believed to form part of an α-Bgt binding receptor because antibodies raised against fusion constructs of ARD can immunoprecipitate[^{125}I]α-Bgt binding activity (Schloss et al., 1988). The amino acid conservation between ARD and the locust non-α subunit suggests that the locust non-α subunit also forms part of an α-Bgt binding protein. However, this locust non-α subunit does not appear to complement the expression of the αL1 subunit in the *X. laevis* oocyte (Marshall et al., in preparation). Interestingly a detailed study of the binding of α-Bgt to *D. melanogaster* has shown two classes of high affinity binding sites. Both sites exhibited a nicotinic cholinergic pharmacology and thus may correspond to two classes of α-Bgt-sensitive nAChRs. The cloning of α and non-α locust subunits together with the possibility of the *D. melanogaster* ALS homologue, still to be cloned in the locust, strongly suggests that a family of nAChR genes are present in the locust nervous system. In order to determine the structure and function of these subtypes further cloning and expression is required. The anatomical distributions of the cloned subunits can then be studied using subunit-specific antibodies and/or *in situ* hybridization methods. The DNA probes generated during this study prove useful in the identification of functionally-distinct types of nicotinic cholinergic synapses and hence lead to a better understanding of how these synapses control behaviour.

ACKNOWLEDGMENTS

We would like to thank Steven Buckingham, Jon David, Ryuso Shingai for their contributions to the electrophysiology experiments. In addition, we are indebted to Leslie Blair, Vince Dionne, Anne Stephenson, George Lunt and Vic Cockcroft for helpful discussions. We are grateful to Guy Tear and Alfonso Martinez-Arias for generously providing the locust genomic library, and to Ms Sue Scott for help in the preparation of the manuscript. John Marshall would like to thank Shell Research, U.K. for support.

REFERENCES

Barnard, E.A., Darlison, M.G., Marshall, J. and Sattelle, D.B. 1989. Structural characterizations of anion and cation channels directly operated by agonists. pp. 159-176 in: "Ion Transport." Kweeling, D. and Benham, C. eds. Academic Press, London..

Benson, J.A. 1988a. Transmitter receptors on insect neuronal somata: GABAergic and cholinergic pharmacology. pp. 193-206 in: "Neurotox 1988: Molecular Basis of Drug and Pesticide Action." G.G. Lunt, ed. Excerpta Medica, Amsterdam.
Benson, J.A., 1988b. Pharmacology of a locust-thoracic ganglion somal nicotinic acetylcholine receptor. pp. 227-240 in: "Nicotinic Receptors in the Nervous System." Clementi, F., Gotti, C. and Sher, E. eds. Springer Verlag, Berlin.
Blair, L.A.C., Levitan, E.S., Marshall, J., Dionne, V.E. and Barnard, E.A. 1988. Single subunits of the receptor form ion channels with properties of the native receptor. Science 242: 577-579.
Bossy, B., Ballivet, M. and Spierer, P. 1988. Conservation of neuronal nicotinic acetylcholine receptors from *Drosophila* to vertebrate central nervous system. EMBO. J. 7: 611-618.
Boulter, J., Connolly, J., Deneris, E., Goldman, D., Heinemann, S. and Patrick, J. 1987. Functional expression of two neuronal nicotinic acetylcholine receptors from cDNA clones identified a gene family. Proc. Natl. Acad. Sci. USA. 84: 7763-7767.
Breer, H., Kleene, R. and Hinz, G. 1985. Molecular forms and subunit structure of the acetylcholine receptor in the central nervous system of insects. J. Neurosci. 5: 3386-3392.
Buckingham, S.D., Sattelle, D.B. and Hue, B. 1990. Synaptic and extrasynaptic actions of bicuculline on identified insect neurones. J. Exp. Biol. in press.
Carr, C.E. and Fourtner. C.R. 1980. Pharmacological analysis of a monosynaptic reflex in the cockroach *Periplaneta americana*. J. Exp. Biol. 86: 259-273.
Chiappinelli, V. A., Hue, B., Mony, L. and Sattelle, D.B. 1989. κ-Bungarotoxin blocks nicotinic transmission at an identified invertebrate synapse. J. Exp. Biol. 141: 61-71.
David, J.A. and Sattelle, D.B. 1984. Actions of cholinergic pharmacological agents on the cell body membrane of the fast coxal depressor motor neurone of the cockroach *Periplaneta americana*. J. Exp. Biol. 108: 119-136.
Deneris, E.S., Connolly, J., Boulter, J., Wada, E., Wada, K., Swanson,L.W., Patrick, J. and Heinemann, S. 1988. Primary structure and expression of β_2: A novel subunit of neuronal nicotinic acetylcholine receptors. Neuron. 1: 45-54.
Freeman, J.A., Schmidt, J.T. and Oswald, R.E. 1980. Effect of α-bungarotoxin in retinotectal synaptic transmission in goldfish and toad. Neurosci. 5: 929-942.
de la Garza, R., Hoffer, B.J. and Freedman, R. 1988. Heterogeneity of nicotinic actions in the rat cerebellum. pp. 887-891 in: "Nicotinic Acetylcholine Receptors in the Nervous System." Clementi, F., Gotti, C. and Sher, E., eds. Springer-Verlag, Heidelberg.
Goldman, D., Deneris, E., Luyten, W., Kochlar, A., Patrick, J. and Heinemann, S. 1987. Members of a nicotinic acetylcholine receptor gene family are expressed in different regions of the mammalian central nervous system. Cell 48: 965-973.
Grenningloh, G., Rienitz, A., Schmitt, B., Methfessel, C., Zensen, M., Beyreuther, K., Gundelfinger, E.D. and Betz, H.. 1987. The strychnine-binding subunit of the glycine receptor shows homology with nicotinic acetylcholine receptors. Nature 328: 215-220.
Hanke, W. and Breer, H. 1986. Channel properties of a neuronal acetylcholine receptor protein purified from the central nervous system of insect reconstituted in planar lipid bilayers. Nature 321: 171-174.
Harrow, I.D., David, J.A. and Sattelle, D.B. 1983. Acetylcholine receptors on identified insect neurones. pp. 12-31 in: "Neuropharmacology of Insects" O'Connor, M. and Whelan, J., eds. Pitman, London.
Harrow, I.D. and Sattelle, D.B. 1982. Acetylcholine receptors on the cell body membrane of giant interneurone 2 in the cockroach *Periplaneta americana*. J. Exp. Biol. 105: 339-350.
Hermans-Borgmeyer, I., Zopf, D., Ryseck, R.P., Hovemann, B., Betz, H. and Gundelfinger, E.D. 1986. Primary structure of a developmentally regulated nicotinic acetylcholine receptor from *Drosophila*. EMBO. J. 5: 1503-1508.
Hoyle, G. and Burrows, M. 1973a. Neural mechanisms underlying behavior in the locust *Schistocerca gregaria*. I. Physiology of identified motor neurons in the metathoracic ganglion. J. Neurobiol. 4: 3-10.
Hoyle, G. and Burrows, M. 1973b. Neural mechanisms underlying behavior in the locust *Schistocerca gregaria*. II. Integrative activity in metathoracic neurons. J. Neurobiol. 4: 43-53.
Kao, P.N. and Karlin, A. 1986. Acetylcholine receptor binding site contains a disulphide crosslink between adjacent half-cystinyl residues. J. Biol. Chem. 261: 8085-8090.

Kao, P.N., Dwark, A.J., Kaldany, R.J., Silver, M.L., Widerman, J., Stein, S. and Karlin, A. 1984. Identification of the α-subunit half-cysteine specifically labelled by an affinity reagent for the acetylcholine receptor binding site. J. Biol. Chem. 259: 1162-1168.

Lane, N.J., Swales, L.S., David, J.A. and Sattelle, D.B. 1982. Differential accessibility to two insect neurones does not account for differences in sensitivity to α-bungarotoxin. Tissue Cell 14: 489-500.

Lees, G., Beadle, D.J. and Botham, R.P. 1983. Cholinergic receptors on cultured neurones from the CNS of embryonic cockroach. Brain Res. 288: 49-59.

Marshall, J., Darlison, M.G., Lunt, G.G. and Barnard, E.A. 1988a. Cloning of a putative nicotinic acetylcholine receptor gene from locust. Biochem. Soc. Trans. 16: 463.

Marshall, J., David, J.A., Darlison, M.G., Barnard, E.A. and Sattelle, D.B. 1988b. Pharmacology, cloning and expression of insect nicotinic acetylcholine receptors. pp 257-281 in: "Nicotinic Acetylcholine Receptors in the Nervous System." Clementi, F., Gotti, C., and Sher, E., eds. Springer-Verlag, Heidelberg.

Nef, P., Onegser, C., Alliod, C., Couturiers, S. and Ballivet, M. 1988. Genes expressed in the brain define three distinct neuronal nicotinic acetylcholine receptors. EMBO. J. 7: 595-601.

Numa, S. 1986. Molecular basis for the function of ion channels. pp. 119-143 in: "Molecular Neurobiology." Kay, J., ed. Biochemical Society, London.

Pinnock, R.D., Lummis, S.C.R., Chiappinelli, V.A. and Sattelle, D.B. 1988. Actions of potent cholinergic anthelminitics (morantel, pyrantel and levamisole) on an identified insect neurone reveal pharmacological differences between nematode and insect acetylcholine receptors. Neuropharm. 27: 843-848.

Raftery, M.A., Hunkapiller, M.W., Strader, C.D. and Hood, L.E. 1980 Acetylcholine receptor: Complex of homologous subunits. Science 208: 1454-1457.

Robertson, R.M. 1986. Neuronal circuits controlling flight in the locust: central generation of rhythm. TINS 9: 278-281.

Sattelle, D.B. 1980. Acetylcholine receptors of insects. Adv. Insect Physiol. 15: 215-315.

Sattelle, D.B. and David, J.A. 1983. Voltage dependent block by histrionicotoxin of the acetylcholine-induced currents in an insect motorneurone cell body. Neurosci. Lett. 43: 37-41.

Sattelle, D.B., Harrow, I.D., Hue, B., Pelhate, M., Gepner, J.I. and Hall, L.M. 1983. α-Bungarotoxin blocks excitatory neurotransmission between cercal sensory neurones and giant interneurone 2 of the cockroach *Periplaneta americana*. J. Exp. Biol. 107: 473-489.

Sattelle, D.B. 1988. Synaptic and extrasynaptic neuronal nicotinic receptors of insects pp. 563-582, in: "The Molecular Basis of Drug and Pesticide Action." Lunt, G. C., ed. Elsevier Biomedical Press, Amsterdam.

Schmieden, V., Grenningloh, C., Schofield, P.R. and Betz, H. 1989. Functional expression in *Xenopus* oocytes of the strychnine binding 48kd subunit of the glycine receptor. EMBO. J. 8: 695-700.

Schofield, P.R., Darlison, M.C., Fujita, N., Burt, D.R., Stephenson, F.A., Rodriguex, H., Rhee, L.M., Ramachandran, J., Reale, V., Glencorse, T.A., Seeburg, P.H. and Barnard, E.A. 1987. Sequence and functional expression of the GABAA receptor shows a ligand-gated receptor superfamily. Nature 328: 221-227.

Stephenson, F.A. 1988. Understanding the $GABA_A$ receptor: a chemically gated ion channel. Biochem. J. 249: 21-32.

Wafford, K.A. and Sattelle, D.B. 1989. L-glutamate receptors on the cell body membrane of an identified insect motor neurone. J. Exp. Biol. 144: 449-462.

Whiting, P. and Lindstrom, J. 1987. Purification and characterization of a nicotinic acetylcholine receptor from rat brain. Proc. Natl. Acad. Sci. USA 84: 595-600.

MOLECULAR ASPECTS OF JUVENILE HORMONE RECEPTION AND ACTION

Lynn M. Riddiford and Subba Reddy Palli

Department of Zoology
University of Washington
Seattle, Washington 98195

INTRODUCTION

Molting and metamorphosis in insects are governed by two hormones, ecdysone which initiates and coordinates the molting process, and juvenile hormone (JH) which allows molting but prevents metamorphosis. In this morphogenetic action JH does not act by itself but rather directs the action of ecdysone. At the molecular level ecdysone can activate or inactivate genes either directly or via the induction of trans-acting regulatory factors (Lepesant and Richards, 1989). During a molt it causes the suppression of ongoing intermolt gene expression and "selects", in a sequential manner, genes necessary for formation of the next stage; the activation of these genes occurs as the ecdysteroid titer falls (Riddiford et al., 1986, 1990). The presence of JH allows the inactivation of the intermolt genes but ensures that they may be re-expressed when the ecdysteroid titer falls. Its presence also prevents the activation of new genes and therefore metamorphosis.

To study this morphogenetic action of JH, we have utilized the larval epidermis of the tobacco hornworm, *Manduca sexta*. This epidermis produces lamellate endocuticle as the larva grows in the intermolt; then, during the molt, endocuticle production ceases while a new epicuticle is deposited (Wolfgang and Riddiford, 1981; 1986). Endocuticle production resumes before ecdysis as the ecdysteroid titer falls. On the final day of feeding in the 5th larval instar, the lamellar structure of the cuticle changes due to the synthesis of several new cuticular proteins. During wandering on the following day, lamellate endocuticle deposition ceases.

The expression of the gene encoding a 14 kDa endocuticular protein (LCP14) (Rebers and Riddiford, 1988) is typical of several larval intermolt endocuticle genes (Riddiford et al., 1986): high during the intermolt when the ecdysteroid titer is low and undetectable during the molt when the ecdysteroid titer is high. LCP 14 is first expressed in the embryo when the first instar cuticle is being deposited (Nagy and Riddiford, unpublished), then in each larval intermolt. When the epidermis is pupally committed by the small rise of ecdysteroid acting in the absence of JH on the final day of feeding, the LCP-14 gene becomes permanently inactivated and is never expressed in either the pupa or adult.

By contrast, the gene family coding for the new 16 and 17 kDa cuticular proteins (LCP16/17) that appear on the final day of feeding is not expressed previously during larval life (Horodyski and Riddiford, 1989). Its activation requires exposure to a low level of 20-hydroxyecdysone (20HE) in the absence of JH equivalent to the hormonal milieu on day 2 of the final instar. Thus, its activation is like that of a gene first expressed at metamorphosis. Once turned on, however, it behaves as a larval intermolt gene in that expression continues either in the absence or presence of JH (Wolfgang and Riddiford, 1986), ceases at wandering, and is not found in either the pupa or adult (Horodyski and Riddiford, 1989).

Molecular Insect Science, Edited by
H. H. Hagedorn *et al.*, Plenum Press, New York, 1990

A NUCLEAR RECEPTOR FOR JH

When *Manduca* epidermis is incubated with labeled JH, 32% of that which enters the cell is bound in the nucleus by two receptors of differing affinities (Osir and Riddiford, 1988). The high affinity receptor has a K_D of 6.6 nM for JH I and 3.8 nM for iodovinylmethoprenol (IVMA), a biologically active dodecadienoate analog of JH (Riddiford et al., 1987); the second receptor has about a 13-fold lower affinity. In spite of similar binding characteristics, the natural JH's were unable to compete with IVMA for its binding and the dodecadienoate JH analogs were unable to compete with JH I for its binding. These results suggested that either there are two different receptors in the nucleus or there are two distinct binding sites on the receptor, either of which could activate the receptor.

To resolve these problems and to isolate the putative JH receptor, we used photoaffinity analogs of JH I and II and of methoprene (Palli et al., 1990). When incubated with day 1 fifth instar epidermal nuclei or with a 0.5 M KCl nuclear extract, the diazoacetate analogs of JH I and II, EBDA and EHDA respectively, covalently labeled a 29 kDa protein. No other protein was labeled in either intact or solubilized nuclei. This binding could be prevented by 250-fold excess JH I or II, but not by methoprene. The diazoketone analog of methoprene, MDK, also labeled a 29 kDa protein but excess methoprene, MDK, or JH I did not prevent the binding.

When the photoaffinity-labeled proteins were separated on 2D gels, EHDA labeled only one 29 kDa protein of pI 5.8; this labeling was prevented by excess JH II. By contrast, MDK labelled the pI 5.8 protein and a second, less abundant protein of pI 6.0. Excess methoprene prevented MDK binding to the pI 6.0 protein but had little effect on its binding to the pI 5.8 protein. Two proteins with the same properties were also found in day 1 fifth instar fat body. Thus, there appear to be two specific 29 kDa JH binding proteins in the nucleus, one that binds the natural homologs and one that binds the dodecadienoate analogs. Whether these two binders are different proteins or differently charged forms of the same protein awaits their isolation and characterization. The reason for the apparent nonspecific binding of MDK to the protein that specifically binds the natural JH homologs is unknown, but we have taken advantage of this binding and the higher specific activity of MDK in the developmental studies described below.

When incubated with the cytosolic (100,000 g supernatant) fraction, all three photoaffinity analogs covalently labeled a 38 kDa protein in addition to a 29 kDa protein (Palli et al., 1990). In contrast to the 29 kDa protein, labeling of the 38 kDa protein by any of the three analogs could be prevented by 250-fold excess of either the natural homologs or of methoprene. This lack of selectivity suggests that this molecule may be an intracellular carrier for these lipophilic molecules similar to that of the cellular retinoic acid binding protein (Chytil, 1984).

DEVELOPMENTAL REGULATION OF JH RECEPTOR LEVELS

Initial studies of IVMA binding by isolated epidermal nuclei showed that specific high affinity binders were present in those from day 1 or day 2 5th instar larvae and from pupae but not in those from wandering stage larvae (Riddiford et al., 1987). Moreover, the loss of the IVMA binders occurred when day 2 epidermis was incubated with 1 μg/ml 20HE for 24 hrs, but not when the tissue was incubated in hormone-free medium. Similarly, MDK labeled a 29 kDa protein in epidermal nuclei from day 1 and 2 larvae and from pupae, but no proteins in nuclei from wandering stage epidermis (Palli et al., 1990). Incubation of day 2 epidermis with 1 μg/ml 20HE for 24 hrs caused the loss of this MDK-binding protein whereas it was retained in tissue incubated in the absence of hormone or with 20HE in the presence of 1 μg/ml JH I (Palli, Hiruma, and Riddiford, unpublished). Thus, when larval epidermis is exposed to 20HE in the absence of JH and becomes pupally committed, it loses its specific nuclear receptors for JH.

When larvae are allatectomized during the final larval molt after the critical period for JH for determination of metamorphosis, they molt to black final instar larvae (Kiguchi and Riddiford, 1978). Three days after allatectomy on day 1 of the 5th instar, the epidermal nuclei showed no specific binding of JH I and little or no 29 kDa MDK-binding protein (Palli, Hiruma, Osir, and Riddiford, unpublished). The 29 kDa binding protein also was not present after pupal ecdysis. Application of 1 μg JH I on the day after wandering caused the

reappearance of the 29 kDa protein. These data suggest that the reappearance of the JH receptors in pupal epidermis is due to the prepupal rise of ecdysteroid in the presence of JH.

JH REGULATION OF LARVAL CUTICLE GENES

Preliminary studies showed that the 29-kDa JH binding protein bound to DNA cellulose. Therefore, it was of interest to determine whether this putative JH receptor might bind to specific genes regulated by JH. As discussed above, both LCP 14 and LCP 16/17 genes are regulated by JH, albeit quite differently. LCP 14 is expressed beginning in the embryo and JH acts to prevent its permanent inactivation during the larval molts, whereas activation of LCP16/17 is suppressed by JH. Once activated by ecdysteroid acting in the absence of JH, it then behaves as LCP14 and can be permanently inactivated by ecdysteroid in the absence of JH.

When the 0.5M KCl-soluble nuclear proteins from day 1 5th instar epidermis were separated by SDS gel electrophoresis and transferred to nitrocellulose, both the LCP 14 and LCP 16/17 genes bound to a 29 kDa protein (Palli et al., 1990). This binding was inhibited by 100-fold excess homologous DNA but not by 1000-fold excess herring sperm DNA. Neither pUC 18 nor part of the coding region of the *Manduca* dopa decarboxylase gene including two introns was bound. Moreover, the 29 kDa protein which bound LCP 14 was present in epidermal nuclei from day 1 5th instar larvae and from day 1 pupae but not in those from allatectomized day 1 or wandering stage larvae. This temporal pattern of expression was the same as that found for the 29-kDa JH binding protein, indicating that they might be the same protein. Confirmation of this hypothesis awaits the purification of the JH-binding protein.

Initial attempts to define the regions of the LCP14 gene which were binding to the 29-kDa protein indicated that there were several, both in the 5' flanking region and in the gene itself. Gel retardation studies thus far show that two adjacent regions of 200 and 400 base pairs respectively near the 5'-end of the first intron bind to nuclear extracts from both day 1 5th and pupal epidermis, but not to those from wandering stage larvae (Palli and Riddiford, unpublished). By contrast, nuclear extracts from epidermis of allatectomized larvae only bind to the 400 base pair piece and not to the 200 base pair piece. Thus the developmental pattern of binding to this latter piece is the same as that of the 29 kDa DNA binding protein. Moreover, preliminary electrophoretic separation of the proteins bound to the retarded DNA indicate that this 5'-most 200 base pair piece is associated with a 29 kDa protein whereas the adjacent 400 base pair region is bound by a 43 kDa protein. Further analysis should reveal the critical sequences necessary for binding.

ARE JH AND RETINOIC ACID RECEPTORS SIMILAR?

Retinoic acid acts as a morphogen in early vertebrate development (Thaller and Eichele, 1987) and also prevents terminal differentiation of mammalian epidermis (Kopan et al., 1987). Moreover, retinoic acid has certain structural features similar to JH. When all trans retinoic acid (Aldrich) was assayed for JH activity in the black *Manduca* larval assay (Fain and Riddiford, 1975), it showed sporadic low activity at doses between 3 and 66 nmols. By contrast, two synthetic retinoids SR5942-64 {p-[(E)-2-(5,6,7,8-tetrahydro-5,5-dimethyl-2-naphthyl)-1-propenyl] benzoic acid} and Ro 13-6298 {ethyl p-[(E)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthyl)-1-propenyl] benzoate} when applied in DMSO gave a typical dose-response curve with ED_{50}s of 41 and 61 nmols respectively. Thus, they are 70-100 times less active than JH III in this assay.

As a second approach to the identification of a JH receptor, we screened the *Manduca* genomic library with the DNA and ligand binding regions of the human retinoic acid receptor cDNA (Petkovich et al., 1987) after finding hybridization to *Manduca* genomic DNA (Palli and Riddiford, unpublished). Sequencing of the 1.7 kb cross-hybridizing region of this clone revealed an open reading frame encoding 34 amino acids in which 13 of 14 near its C-terminal end were identical. These 14 amino acids comprise the C-terminal half of the second zinc finger of the human retinoic acid receptor (Petkovich et al., 1987). On either side of this stretch no homologies are seen, but one potential splice site is present. So far we have not found this gene expressed at detectable levels in embryos or in epidermis

during the last two larval instars or in the developing pupa or adult. Therefore, using this 1.7 kb region containing the *Manduca* "zinc finger" as a probe, we rescreened the genomic library and found a second clone that is expressed in the epidermis, particularly during the larval and pupal molts when the ecdysteroid levels are high. Whether this gene codes for either a JH or ecdysone receptor awaits its further characterization.

CONCLUSIONS

Use of photoaffinity analogs of JH I and II and of methoprene have shown that there are two 29 kDa proteins in the nucleus of both larval epidermis and fat body of *Manduca* that bind these compounds. The pI 5.8 protein is specific for the natural JH homologs and nonspecifically binds MDK, whereas the pI 6.0 protein binds only the dodecadienoate analog. This 29 kDa JH-binding protein is present in both larval and pupal epidermis, but not in that from wandering stage or allatectomized larvae, thus showing the same developmental pattern as that found for the high affinity JH binders. Moreover, this same developmental pattern is found for a 29 kDa nuclear protein which binds to the DNA of two larval cuticle genes whose expression is regulated by JH. Thus, we hypothesize that the nuclear 29 kDa JH-binding protein is a JH receptor that binds to DNA and thus can modulate ecdysteroid action. Moreover, its apparent specific binding to a specific 200 base pair region in the first intron of the LCP14 gene indicates that it may act directly on larval genes. Once either the JH-binding protein is purified or the gene encoding it is isolated, we will be able to test this hypothesis directly.

ACKNOWLEDGMENTS

We thank Dr. Marcia Dawson of Stanford Research Institute for the retinoids. The unpublished work was supported by NSF DCB 88-18876.

REFERENCES

Chytil, F. 1984. Retinoic acid: Biochemistry, pharmacology, toxicology, and therapeutic use. Pharm. Rev. 36: 93S-100S.

Fain, M.J. and Riddiford, L. M. 1975. Juvenile hormone titers in the hemolymph during late larval development of the tobacco hornworm, *Manduca sexta*. Biol. Bull. 149: 506-521.

Horodyski, F. M. and Riddiford, L. M. 1989. Expression and hormonal control of a new larval cuticular multigene family at the onset of metamorphosis of the tobacco hornworm. Devel. Biol. 132: 292-303.

Kiguchi, K. and Riddiford, L. M. 1978. The role of juvenile hormone in pupal development of the tobacco hornworm, *Manduca sexta*. J. Insect Physiol. 24: 673-680.

Kopan, R., Trask, G., and Fuchs, E. 1987. Retinoids as important regulators of terminal differentiation: Examining keratin expression in individual epidermal cells at various stages of keratinization. J. Cell Biol. 105: 427-440.

Lepesant, J.-A. and Richards, G. 1989. Ecdysteroid-regulated genes. pp. 355-367 in: "Ecdysone. From Chemistry to Mode of Action." J. Koolman, ed. Georg Thieme, Stuttgart.

Osir, E.O. and Riddiford, L.M. 1988. Nuclear binding of juvenile hormone and its analogs in the epidermis of the tobacco hornworm. J. Biol. Chem. 263: 13812-13818.

Palli, S.R., Osir, E.O., Eng, W-s., Boehm, M. F., Edwards, M., Kulcsar, P., Ujvary, I., Hiruma, K., Prestwich, G.D. and Riddiford, L. M. 1990. Juvenile hormone receptors in larval insect epidermis: Identification by photoaffinity labeling. Proc. Nat. Acad. Sci. U.S.A. 87: 796-800.

Petkovich, M., Brand, N. J., Krust, A., and Chambon, P. 1987. A human retinoic acid receptor which belongs to the family of nuclear receptors. Nature 330: 444-450.

Rebers, J. E. and Riddiford, L.M. 1988. Structure and expression of a *Manduca sexta* larval cuticle gene homologous to *Drosophila* cuticle genes. J. Mol. Biol. 203: 411-423.

Riddiford, L. M., Baeckmann, A., Hice, R. H., and Rebers, J. 1986. Developmental expression of three genes for larval cuticular proteins of the tobacco hornworm,

Manduca sexta. Devel. Biol. 118: 82-94.
Riddiford, L.M., Osir, E.O., Fittinghoff, C.M., and Green, J. M. 1987. Juvenile hormone analogue binding in *Manduca* epidermis. Insect Biochem. 17: 1039-1043.
Riddiford, L. M., Palli, S. R. and Hiruma K. 1990. Hormonal control of sequential gene expression in *Manduca* epidermis. in: "Progress in Comparative Endocrinology." A. Epple, ed. Alan R. Liss, New York. In press.
Thaller, C. and Eichele, G. 1987. Identification and spatial distribution of retinoids in the developing chick limb bud. Nature 327: 625-628.
Wolfgang, W. J. and Riddiford, L. M. 1981. Cuticular morphogenesis during continuous growth of the final instar larva of a moth. Tiss. Cell 13: 757-772.
Wolfgang, W. J. and Riddiford, L. M. 1986. Larval cuticular morphogenesis in the tobacco hornworm, *Manduca sexta*, and its hormonal regulation. Devel. Biol. 113: 305-316.

DEVELOPMENTAL REGULATION OF SILK GENE EXPRESSION IN *Bombyx mori*

Yoshiaki Suzuki, Shigeharu Takiya, Toshiharu Suzuki, Chi-chung Hui,
Kenji Matsuno, Masakazu Fukuta, Toshifumi Nagata, and Kohji Ueno

Department of Developmental Biology
National Institute for Basic Biology
Myodaiji-cho, Okazaki 444, Japan

INTRODUCTION

Our major concern has been understanding how cells are specialized and express a set of genes during development. Along this line, our target has been a dissection of silk gene regulation in the silk gland of *Bombyx mori* (Suzuki, 1977; Suzuki et al., 1987). The development of the silk gland originates as an invagination of the ectoderm in the labial segment of stage 19 embryos, and completes morphologically by stage 25 (Nunome, 1937). Transcription of the fibroin (heavy chain) gene (Suzuki and Brown, 1972; Suzuki et al., 1972; Ohshima and Suzuki, 1977; Tsujimoto and Suzuki, 1979a, b) begins at around stage 25 of the embryos (Ohta et al., 1988). After this first activation the fibroin gene is repeatedly switched on and off in the posterior silk gland cells during larval development (Suzuki and Suzuki, 1974; Suzuki and Giza, 1976; Maekawa and Suzuki, 1980). When fibroin gene transcription in the posterior silk glands of the fifth instar larvae was analyzed by nuclear run-on assays, it was found that the transcription is restricted to the anterior region at the beginning of the instar and spreads toward the posterior region as the stage proceeds (Obara and Suzuki, 1988). Transcription of the fibroin light chain (or P25) gene was found to occur in parallel with that of the fibroin gene during the fourth molting stage and the fifth larval instar (Couble et al., 1983; Kimura et al., 1985). Transcription of the sericin-1 gene (Okamoto et al., 1982), one of the glue protein genes expressed mostly in the posterior region of the middle silk gland, was weakly detected in the third and fourth larval instars, turned off during the fourth moulting stage, and turned on again in the fifth larval instar reaching high activity toward the late fifth instar (Ishikawa and Suzuki, 1985; Hamada et al., 1987; Obara and Suzuki, 1988). In contrast, the sericin-2 gene is expressed in the anterior region of the middle silk gland (Couble et al., 1987). These circumstances indicate that the silk gland is a good model system for the study of temporal and spatial gene regulation in development (Suzuki, 1977; Suzuki et al., 1987).

Specific gene amplification, as well as gene structure alteration including methylation modification during the process of cell differentiation, has been ruled out for the fibroin gene (Suzuki et al., 1972; Suzuki and Adachi, 1984; Tsujimoto and Suzuki, 1984). Expression of the fibroin gene is mostly controlled at the level of transcription (Suzuki and Suzuki, 1974; Suzuki and Giza, 1976; Obara and Suzuki, 1988). To analyze the underlying mechanisms controlling transcription of the fibroin gene in the posterior silk gland, and the sericin-1 gene in the middle silk gland, a major strategy we have been using is to develop and utilize cell-free transcription systems and to characterize the regulatory factors involved (Suzuki et al., 1987). Through the characterization of these factors and their genes, the molecular events involved in the process of cell specialization will be clarified.

Molecular Insect Science, Edited by
H. H. Hagedorn *et al.*, Plenum Press, New York, 1990

DEVELOPMENT OF CELL-FREE TRANSCRIPTION SYSTEMS

Use of cell-free transcription systems is a powerful approach for analysis of transcription regulation mechanisms of tissue-specific genes and the nature of the factors involved in their regulation (Suzuki et al., 1987). Since the first establishment of tissue extracts that can transcribe the fibroin gene faithfully (Tsuda and Suzuki, 1981), efforts have been made to develop and improve cell-free transcription systems from various sources (Gorski et al., 1986; Mizushima-Sugano and Roeder, 1986; Suzuki et al., 1986; Wolffe and Brown, 1987; Biggin and Tjian, 1988; Corthesy et al., 1988; Soeller et al., 1988; Suzuki et al., 1990). Increasing numbers of extracts from various sources have become available in which a preferential transcription of tissue-specific genes as well as homeotic genes is possible.

For the *B. mori* system, 14 kinds of cell-free transcription systems that can transcribe the fibroin gene faithfully have been developed (Tsuda and Suzuki, 1981; Suzuki et al., 1986; Takiya et al., 1990; Suzuki et al., 1990; see Table 1). Use of these systems demonstrates the specificity of the cell-free transcription of the fibroin and sericin-1 genes (Suzuki et al., 1986; Matsuno et al., 1989b; Suzuki et al., 1990). Recently, a differential transcription of the fibroin gene and the sericin-1 gene has been attained in silk gland extracts (Suzuki et al., 1990); in posterior silk gland extracts the fibroin gene was transcribed preferentially relative to the sericin-1 gene, and in the middle silk gland extracts the sericin-1 gene was transcribed more efficiently than the fibroin gene. These results open a way to characterize the factors responsible for the differential transcription of these genes.

Table 1. Cell-free Transcription Systems from *B. mori* Sources

SOURCES	STAGES		REFERENCES
Embryos		Stage 17	Suzuki et al., 1990
		Stage 19	"
		Stage 24	"
		Stage 25	"
Post. silk gland	5th instar	1.0-day-old	"
		1.5-day-old	"
		2.0-day-old	Tsuda and Suzuki, 1981
		2.5-day-old	Suzuki et al., 1990
Middle silk gland	5th instar	2.0-day-old	Tsuda and Suzuki, 1981
		3.0-day-old	Suzuki et al., 1990
		4.0-day-old	"
		5.0-day-old	"
Ovarian tissues	Pupal	4.0-day-old	Suzuki et al., 1986
Cell line e21	Late embryonic stage		"

ASSIGNMENT OF *CIS*-ACTING ELEMENTS OF THE FIBROIN AND SERICIN-1 GENES IN CELL-FREE TRANSCRIPTION SYSTEMS

Using these cell-free systems and a series of deletion mutants, *cis*-acting elements of the fibroin and sericin-1 genes have been identified (Tsuda and Suzuki, 1983; Suzuki et al., 1986; Hui and Suzuki, 1989; Matsuno et al., 1989b; Takiya et al., 1990). About 40 bp sequences covering the TATA box region and the transcription initiation region of the fibroin gene were found to be the basic promoter (Tsuda and Suzuki, 1981; Tsujimoto et al., 1981; Takiya et al., 1990). This region (the core-promoter; CP in Fig. 1) serves not only as a general basic promoter but also as a region which exerts the fibroin gene-specific transcription (Takiya et al., 1990). About 200 bp sequences upstream of the TATA box region (enhancer-I; ENI) and a part of the intron region (enhancer-II; ENII) have been identified to have an enhancer-like function (Tsuda and Suzuki, 1983; Hui and Suzuki, 1989; Takiya et al., 1990). The upstream sequences can be divided into at least two parts; the distal upstream element (-238 to -73) and the proximal upstream element (-72 to -32) (Suzuki et al., 1986). The distal upstream element, which accommodates an enhancer-like function and

corresponds to ENI (Tsuda and Suzuki, 1983; Hui and Suzuki, 1989), is recognized most efficiently and specifically by the factors in the posterior silk gland extracts and stimulate the transcription from the basal promoter (Suzuki et al., 1986; Tsuda et al., 1986; Suzuki et al., 1990). This stimulation is very specific to the posterior silk gland extracts and is not detectable in most of the non-posterior silk gland extracts with the exception of the middle silk gland extract obtained from 2-day-old fifth instar larvae which gives a weak stimulation (Suzuki et al., 1986; Suzuki et al., 1990). Although the meaning of this weak stimulation is not known, the overall results mentioned above indicate that these cell-free systems are reflecting the *in vivo* regulation.

At least three elements were identified for the sericin-1 gene (Matsuno et al., 1989b); at around -200, -100, and the TATA box region. As described in the next section the sequence between -204 and -183 is one of the sites protected by protein binding; the SC site (see Fig. 1; Matsuno et al., 1989b). A multimer of the SC element, when introduced in the upstream of the sericin-1 promoter (pSr' Δ-38), stimulated the transcription of this promoter (K. Matsuno et al., submitted).

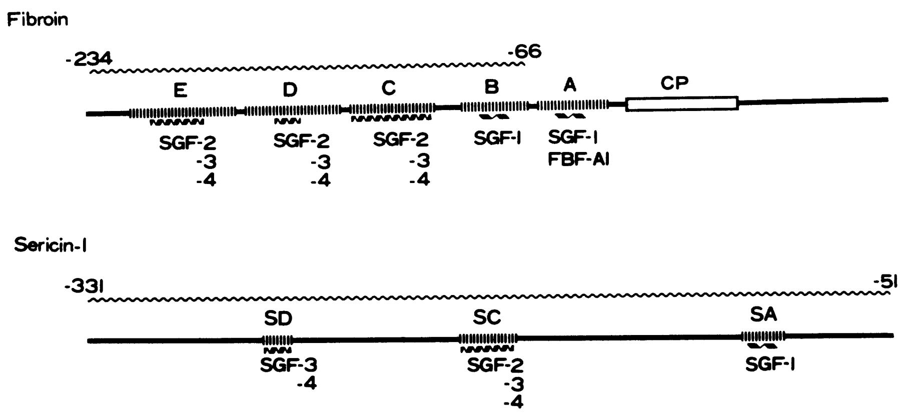


Fig. 1. Protein Binding Sites in the Fibroin and Sericin-1 Upstream Elements (Hui and Suzuki, 1989). The known protein binding sites (striped boxes in these two promoters are summarized. A to E in the figure corresponds to FA to FE in the text, respectively. The wavy lines represent the upstream elements. The symbols ( ) and ( ) indicate the "TGTTT" motif for SGF-1, and the "TCAATTAAAT" motif for a number of *Drosophila melanogasster* homeodomain proteins (Hoey and Levine, 1988), respectively. The types of posterior silk gland factors that interact with these binding sites are listed.

CHARACTERIZATION OF *TRANS*-ACTING FACTORS

To detect *trans*-acting factors and assign the binding sites of these factors on the fibroin and sericin-1 genes, gel shift assays and nuclease protection experiments have been carried out. Among several gel shift complexes formed with a DNA probe (-234/-66) and nuclear extracts, one complex was found to be specific to the posterior silk gland extracts (Suzuki and Suzuki, 1988). DNase I footprinting assay on the complex revealed protection sites at -180/-177, -167/-155, and -120/-110. Fractionation of the posterior silk gland nuclear proteins indicated that the complex is constructed from two proteins; one (FFI) having a weak DNA binding ability and the other (FFII) with virtually no DNA binding ability (Suzuki and Suzuki, 1988; unpublished results). Therefore, the complex formation includes not only a protein-DNA interaction but also a protein-protein interaction. Both proteins are being partially purified and characterized (Suzuki and Suzuki, unpublished). The function of these proteins in transcription remains unknown. The same or independent protein-protein interaction at the upstream region of the fibroin gene was detected with a new protein-mediated blotting method (Matsuno et al., 1989a).

Footprinting analysis with silk gland nuclear extracts revealed several protection sites in the upstream regions of the fibroin gene and the sericin-1 gene (see Fig. 1); for the fibroin

gene, FE (-220/-180), FD (-170/-135), FC (-135/-100), FB (-90/-60), FA (-60/-35), the TATA box region and the transcription initiation region (CP), and a part of the intron (C.-c. Hui and Y. Suzuki, submitted; Takiya et al., 1990; S. Takiya, unpublished), and for the sericin-1 gene, SC (-204/-183), SB (-149/-135), and SA (-103/-85) (Matsuno et al., 1989b). The SB site was a weak protection site, and not shown in Fig. 1. Later, an additional site SD was detected and shown in Fig. 1. Factors binding to these sites have been characterized and named SGF-1 to -4 and FBF-A1 (Fig. 1). Most of these sites are important for the transcriptional enhancement of the basal promoter activity of the fibroin gene (Tsuda and Suzuki, 1983; Suzuki et al., 1986; Tsuda et al., 1986; Takiya et al., 1990; C.-c. Hui and Y. Suzuki, submitted) and the sericin-1 gene (Matsuno et al., 1989b; K. Matsuno et al., submitted). Some of the individual sites like FE and SC are known to have transcription enhancing function (C.-c. Hui et al., submitted; K. Matsuno et al., submitted).

ARE HOMEO DOMAIN PROTEINS INVOLVED IN THE SILK GLAND DIFFERENTIATION PROCESSES AND/OR THE SILK GENE EXPRESSION?

Repeats of TCAATTAAAT or its variants are detected in FE, FD, and FC regions including the one at -167/-158. Interestingly, this consensus sequence is identical with that of the *Drosophila melanogasster engrailed* gene bound with some of the homeo box proteins carrying a helix-turn-helix structure (Desplan et al., 1988: Hoey and Levine, 1988). Binding and protection of the homeodomain proteins on these consensus sequences of the fibroin gene have been demonstrated, and proteins in the posterior silk gland nuclear extracts protected the consensus sequences of the *D. melanogasster engrailed* gene (C.-c. Hui and Y. Suzuki, unpublished). These observations raised an important question. Could some of these proteins binding to the upstream regions of the fibroin and sericin-1 genes be homeo box proteins? If this were the case, it would suggest an important developmental feature that tissue differentiation and/or tissue-specific gene transcription could also be controlled by some homeo box containing genes. Recently, similar cases have been reported for tissue-specific genes that function in the pituitary gland (Bodner and Karin, 1987; Bodner et al., 1988) and lymphocytes (Ko et al., 1989; Muller et al., 1988; Scheidereit et al., 1989); a new class of regulatory genes containing POU domain sequences which reveal a weak homology with the homeo domains was discovered. The importance of the MyoD protein, which belongs to another regulatory protein member carrying a helix-loop-helix structure in the differentiation process of myogenic cells, has also been reported (Davis et al., 1987). The gel shift assay coupled with mutagenesis of oligonucleotides of the fibroin and sericin-1 upstream regions suggest that some homeodomain proteins are involved in binding to these regions (C.-c. Hui et al., submitted). Cloning of cDNAs from the silk glands has revealed that the *B. mori* homologs of *Antennapedia, engrailed, invected,* and others (C.-c. Hui, unpublished) as well as POU domain containing genes (Fukuta, unpublished) are expressed in silk glands.

Characterization of these factors and their genes would clarify how differential transcription of the fibroin and sericin-1 genes is controlled in these differentiated tissues. These analyses would at the same time give us a clue to understand what kind of molecules are involved in the differentiation processes. The cell-free transcription systems developed here are a key tool for the molecular analyses.

GENOMIC HOMEO BOX CONTAINING GENES

In *B. mori* more than 20 homeotic mutants have been reported (see Tazima, 1964). Assuming that these homeotic genes may contain homeo box sequences, we have begun cloning genomic DNA fragments containing homeo box sequences using *D. melanogasster* homeo box sequences as probes (Suzuki et al., 1987). *B. mori* homologs of *Antennapedia* (W. Hara, unpublished), *engrailed,* and *Sex comb reduced* (K. Ueno, unpublished) have been cloned and their identities have been confirmed by partial sequence analysis. Developmental expression of the *Antennapedia* homolog has been analyzed in the embryonic stages as well as the larval stages (W. Hara et al., unpublished). Further characterization of these and others that are expected is important for the understanding of the control of body plan as well as control of cell differentiation including silk gland development and silk gene regulation.

Further details of our recent experiments can be found in: Hui and Suzuki, Develop. Growth Differ., in press (1990); Hui et. al., J. Mol. Biol., in press (1990a); Hui et. al., J. Mol. Biol., in press (1990b); and Matsumo et. al., Nucl. Acids Res., in press (1990).

REFERENCES

Biggin, M. D. and Tjian, R. 1988. Transcription factors that activate the *Ultrabithorax* promoter in developmentally staged extracts. Cell 53: 699-711.

Bodner, M. and Karin, M. 1987. A pituitary-specific *trans*-acting factor can stimulate transcription from the growth hormone promoter in extracts of nonexpressing cells. Cell 50: 267-275.

Bodner, M., Castrillo, J.-L., Theill, L. E., Deerinck, T., Ellisman, M., and Karin, M. 1988. The pituitary-specific transcription factor GHF1 is a homeobox-containing protein. Cell 55: 505-518.

Corthesy, B., Hipskind, R., Theuraz, I., and Wahli, W. 1988. Estrogen-dependent *in vitro* transcription from the vitellogenin promoter in liver nuclear extracts. Science 239: 1137-1139.

Couble, P., Moine, A., Garel, A., and Prudhomme, J. C. 1983. Developmental variations of a non-fibroin mRNA encoding for a low molecular weight silk protein. Develop. Biol. 97: 398-407.

Couble, P., Michaille, J.-J., Garel, A., Couble, M.-L., and Prudhomme, J.-C. 1987. Developmental switches of sericin mRNA splicing in individual cells of *Bombyx mori* silkgland. Develop. Biol. 124: 431-440.

Davis, R. L., Weintraub, H., and Lassar, A. B. 1987. Expression of a single transfected cDNA converts fibroblasts to myoblasts. Cell 51: 987-1000.

Desplan, C., Theis, J., and O'Farrell 1988. The sequence specificity of homeodomain-DNA interaction. Cell 54: 1081-1090.

Gorski, K., Carneiro, M., and Schibler, U. 1986. Tissue-specific *in vitro* transcription from the mouse albumin promoter. Cell 47: 767-776.

Hamada, Y., Yamashita, O., and Suzuki, Y. 1987. Haemolymph control of sericin gene expression studied by organ transplantation. Cell Differ. 20: 65-76.

Hoey, T. and Levine, M. 1988. Divergent homeo box proteins recognize similar DNA sequences in *Drosophila*. Nature 332: 858-861.

Hui, C.-c., and Suzuki, Y. 1989. Enhancement of transcription from the Ad2 major late promoter by upstream elements of the fibroin and sericin-1 genes in silk gland extracts. Gene 85: 405-413.

Ishikawa, E. and Suzuki, Y. 1985. Tissue- and stage-specific expression of sericin genes in the middle silk gland of *Bombys mori*. Develop. Growth Differ. 27: 73-82.

Kimura, K., Oyama, F., Ueda, H., Mizuno, S., and Shimura, K. 1985. Molecular cloning of the fibroin light chain complementary DNA and its use in the study of the expression of the light chain gene in the posterior silk gland of *Bombyx mori*. Experientia 41: 1167-1171.

Ko, H.-S., Fast, P., McBride, W., and Staudt, L. M. 1989. A human protein specific for the immunoglobulin octamer DNA motif contains a functional homeobox domain. Cell 55: 135-144.

Maekawa, H. and Suzuki, Y. 1980. Repeated turn-off and turn-on of fibroin gene transcription during silk gland development of *Bombyx mori*. Develop. Biol. 78: 394-406.

Matsuno, K., Suzuki, T., Takiya, S., and Suzuki, Y. 1989a. Complex formation with the fibroin gene enhancer through a protein-protein interaction analyzed by a modified DNA-binding assay. J. Biol. Chem. 264: 4599-4604.

Matsuno, K., Hui, C.-c., Takiya, S., Suzuki, T., Ueno, K., and Suzuki, Y. 1989b. Transcription signals and protein binding sites for sericin gene transcription *in vitro*. J. Biol. Chem. 264: 18707-18713.

Mizushima-Sugano, J. and Roeder, R. G. 1986. Cell-type-specific transcription of an immunoglobulin k light chain gene *in vitro*. Proc. Natl. Acad. Sci. USA 83: 8511-8515.

Muller, M. M., Ruppert, S., Schaffner, W., and Matthias, P. 1988. A cloned octamer transcription factor stimulates transcription from lymphoid-specific promoters in non-B cells. Nature 336: 544-551.

Nunome, J. 1937. The silk gland development of *Bombyx mori*. Bull. Appl. Zool. 9: 68-92 (in Japanese).
Obara, T. and Suzuki, Y. 1988. Temporal and spatial control of silk gene transcription analyzed by nuclear run-on assays. Develop. Biol. 127: 384-391.
Ohshima, Y. and Suzuki, Y. 1977. Cloning of the silk fibroin gene and its flanking sequences. Proc. Natl. Acad. Sci. USA 74: 5363-5367.
Ohta, S., Suzuki, Y., Hara, W., Takiya, S., and Suzuki, T. 1988. Fibroin gene transcription in the embryonic stages of the silkworm, *Bombyx mori*. Develop. Growth & Differ. 30: 293-299.
Okamoto, H., Ishikawa, E., and Suzuki, Y. 1982. Structural analysis of sericin genes. J. Biol. Chem. 257: 15192-15199.
Scheidereit, C., Cromlish, J. S., Gerster, T., Kawakami, K., Balmaceda, C.-G., Currie, R. A., and Roeder, R. G. 1988. A human lymphoid-specific transcription by mammalian RNA polymerase II. Nature 336: 551-557.
Soeller, W. C., Poole, S. J., and Kornberg, T. 1988. *In vitro* transcription of the *Drosophila engrailed* gene. Genes Dev. 2: 68-81.
Suzuki, Y. 1977. Differentiation of the silk gland: A model system for the study of differential gene action. pp. 1-44 in "Results and Problems in Cell Differentiation." Vol. 8. Beermann, W. ed. Berlin/Heidelberg, Springer-Verlag.
Suzuki, Y. and Brown, D. D. 1972. Isolation and identification of the messenger RNA for silk fibroin from *Bombyx mori*. J. Mol. Biol. 63: 409-429.
Suzuki, Y. and Suzuki, E. 1974. Quantitative measurements of fibroin messenger RNA synthesis in the posterior silk gland of normal and mutant *Bombyx mori*. J. Mol. Biol. 88: 393-407.
Suzuki, Y. and Giza, P. E. 1976. Accentuated expression of silk fibroin gene *in vivo* and *in vitro*. J. Mol. Biol. 107: 183-206.
Suzuki, Y. and Adachi, S. 1984. Signal sequences associated with fibroin gene expression are identical in fibroin-producer and -nonproducer tissues. Develop. Growth Differ. 26: 139-147.
Suzuki, T. and Suzuki, Y. 1988. Interaction of composite protein complex with the fibroin enhancer sequence. J. Biol. Chem. 263: 5979-5986
Suzuki, Y., Gage, L. P., and Brown, D. D. 1972. The genes for fibroin in *Bombyx mori*. J. Mol. Biol. 70: 637-649.
Suzuki, Y., Tsuda, M., Takiya, S., Hirose, S., Suzuki, E., Kameda, M., and Ninaki, O. 1986. Tissue-specific transcription enhancement of the fibroin gene characterized by cell-free systems. Proc. Natl. Acad. Sci. USA 83: 9522-9526.
Suzuki, Y., Takiya, S., Hara, W., Obara, T., Suzuki, T., and Hui, C.-c. 1987. Developmental regulation of the tissue-specific genes and the homeotic genes in *Bombyx mori*. pp. 13-26 in "Gunma Symposium Endocrinology." Vol. 24. Inst. Endocrinol., Gunma Univ. ed. Center for Academic Publications Japan, Tokyo and VNU Science Press BV, Utrecht.
Suzuki, Y., Obara, T., Takiya, S., Hui, C.-c., Matsuno, K., Suzuki, T., Suzuki, E., Ohkubo M., and Tamura, T. 1990. Differential transcription of the fibroin and sericin-1 genes in cell-free extracts. Develop. Growth Differ. in press.
Takiya, S., Hui, C.-c., and Suzuki, Y. 1990. A contribution of the core-promoter and its surrounding regions to the preferential transcription of the fibroin gene in posterior silk gland extracts. EMBO J. 9: 489-496.
Tazima, Y. 1964. "The Genetics of the Silkworm." pp. 60-75. Logos Press and Academic Press, London.
Tsuda, M. and Suzuki, Y. 1981. Faithful transcription initiation of fibroin gene in a homologous cell-free system reveals an enhancing effect of 5' flanking sequence far upstream. Cell 27: 175-182.
Tsuda, M. and Suzuki, Y. 1983. Transcription modulation *in vitro* of the fibroin gene exerted by a 200-base-pair region upstream from the "TATA" box. Proc. Natl. Acad. Sci. USA 80: 7442-7446.
Tsuda, M., Hirose, S., and Suzuki, Y. 1986. Participation of the upstream region of the fibroin gene in the formation of transcription complex *in vitro*. Mol. Cell. Biol. 6: 3928-3933.
Tsujimoto, Y. and Suzuki, Y. 1979a. Structural analysis of the fibroin gene at the 5' end and its surrounding regions. Cell 16: 425-436.

Tsujimoto, Y. and Suzuki, Y. 1979b. The DNA sequence of *Bombyx mori* fibroin gene including the 5' flanking, mRNA coding, entire intervening and fibroin protein coding regions. Cell 18: 591-600.
Tsujimoto, Y. and Suzuki, Y. 1984. Natural fibroin genes purified without using cloning procedures from fibroin-producing and nonproducing tissues reveal indistinguishable structure and function. Proc. Natl. Acad. Sci. USA 81: 1644-1648.
Tsujimoto, Y., Hirose, S., Tsuda, M., and Suzuki, Y. 1981. Promoter sequence of fibroin gene assigned by *in vitro* transcription system. Proc. Natl. Acad. Sci. USA 78: 4838-4842.
Wolffe, A. P. and Brown, D. D. 1987. Differential 5S RNA gene expression *in vitro*. Cell 51: 733-740.

REGULATING GENES FOR METAMORPHOSIS: CONCEPTS AND RESULTS

Judith H. Willis

Department of Entomology
University of Illinois
Urbana, IL 61801

INTRODUCTION

The transformation of a drab caterpillar into a spectacular butterfly has fascinated mankind for millennia. That metamorphosis is regulated by only two classes of hormones, ecdysteroids and juvenoids, should excite the interest of biologists jaded by the complexity of embryonic development. While ecdysteroids drive a molt, it is the juvenoids that control the nature of the molt. In their absence metamorphosis ensues, in their presence the molt results in continuance of the "status quo", with the insect forming a new instar similar to the previous one.

The aim of this paper is to report on what we know about how juvenoids exert their "status quo" action to prevent metamorphosis. Since my previous summary (Willis, 1981), our acquisition of knowledge on the molecules involved in metamorphosis has been vast. We now have extensive information on amino acid sequences of cuticular proteins that are used to create the form of each metamorphic stage. Some cuticular protein genes have been isolated and characterized. For the hormones that regulate metamorphosis, we have information on structures and titers, sequences of peptides that regulate their production, and we are beginning to home in on their receptors. The immediate future is promising therefore for resolving issues that have been outstanding for a half-century.

In 1940, V. B.Wigglesworth described studies on *Rhodnius prolixus* that established the principal facts concerning the anti-metamorphic action of juvenile hormone. In this classic paper Wigglesworth documented the role of the corpora allata in inhibiting metamorphosis and introduced the term "juvenile hormone". His studies revealed that the critical period for juvenoid action extends for about half of the twenty-eight day period between the blood meal and ecdysis. The response of the epidermis to juvenile hormone changes during this two week period. Some characters on the new cuticle are adult even when juvenile hormone is present early in the critical period; others remain larval even when the source of juvenile hormone is applied much later. It is evident that Wigglesworth recognized that juvenoid action on a single epidermal cell was not all-or-none, for he illustrated islands of composite cuticle that combined the characteristics of both larva and adult. Later work by Wigglesworth (1970) and others (Lawrence, 1969; Willis and Hollowell, 1976; Willis et al. 1982) amplified this often-ignored finding.

Wigglesworth's 1940 paper also contained the first model for juvenoid action, one that became the prototype for most of the diagrams produced since. Wigglesworth postulated that juvenoids activate larval enzyme systems. His model considers two epidermal cells. In one, adult enzymes are active; in the other, the "inhibitory hormone" (the term juvenile hormone was not introduced until the end of the paper) favors activities by larval enzymes. Over the years there has been a "metamorphosis" of his model: juvenoids went from affecting enzymes, to working at the level of ribosomes, messages, and most recently, genes.

In the half century that has elapsed since this seminal publication, we have learned to use isotopes to monitor macromolecular synthesis and to label hormones so we can identify their targets and their metabolic fate. Thanks to isotopes and radioimmune assays we have

Molecular Insect Science, Edited by
H. H. Hagedorn *et al.*, Plenum Press, New York, 1990

sophisticated techniques for measuring hormone titers. We have developed tissue culture media that allowed us to probe the regulation of the biosynthesis of juvenoids and to establish that juvenoids act directly on the epidermis. Electrophoresis allows us to separate the proteins of cuticle and blood. Now we can apply the entire armamentarium of molecular biology and isolate the genes for these proteins, sequence them, and begin to probe how they are regulated.

It is thus timely to have a status report on the "status quo" action of juvenoids. The format of this report will be to pose a series of precise questions about the way juvenoids regulate metamorphosis, and to analyze recent literature to see what answers are available.

Do unique structures of a metamorphic stage require unique genes? The concept of stage-specific gene sets as originally conceived (Wigglesworth, 1959; Williams and Kafatos, 1971) is certainly incorrect. In brief, our own data on cuticular proteins of *Hyalophora cecropia* (summarized in Willis, 1986, 1990), Riddiford's (1987) data on the cuticular proteins of *Manduca sexta*, and the findings of Henikoff and Eghtedarzadeh (1987) on the cuticular proteins of *Drosophila melanogaster* have established that quite different structures can be built, at least in part, from the same cuticular proteins, and that a single cuticular protein gene can be used in two or more metamorphic stages. One of the so-called "larval" serum proteins, has been shown to be synthesized in adult flies (Shirras and Bownes, 1989). Detailed evidence is accumulating that in *D. melanogaster* even a gene that specifies morphogenetic instructions can be used at multiple stages in development (See Willis, 1990 for details.)

If, as seems apparent, stage-specific sets of genes are not essential to build different metamorphic stages, then juvenoids can not be working by selecting or repressing such sets of genes. So, one must ask, at what level may juvenoids be affecting metamorphosis?

There are three main possibilities for how juvenoids exert their "status quo" action on the epidermis. They could act: 1) by preventing new mRNA synthesis, or 2) by direct binding of JH plus its receptor to cuticular protein genes, or 3) by an indirect action on cuticular protein genes. What information do we have to support or refute each of these three possibilities?

Prevention of new mRNA synthesis

The most obvious way for juvenoids to act would be to prevent the formation of new mRNA so that a cell would have to depend on the use of preformed messages, messages made and used in the previous stage. If this were the way juvenoids worked, we would expect actinomycin D, an effective inhibitor of mRNA synthesis, to mimic the action of juvenoids. Actinomycin D does, in fact, cause the production of second pupae in *Tenebrio molitor*. Several laboratories have documented that it occurs reproducibly in a small fraction of treated animals (Chase, 1967, 1970; Socha and Sehnal, 1972; Andersen et al. 1973; Roberts and Willis, 1980c). At first these actinomycin D-induced second pupae were attributed to the reuse of old mRNAs. Phillips and Loughton (1979) have come up with a far more plausible suggestion, namely that actinomycin D acts to inhibit the appearance of juvenile hormone esterase, and hence persistent juvenoids can exert their "status quo" action.

The rare action of actinomycin D in *T. molitor* thus provides only the weakest support for juvenoids acting by blocking all transcription. There is far stronger evidence against the hypothesis. We know from the work on cuticular proteins of *T. molitor* (Roberts and Willis, 1980 a,b), of *M. sexta* (Kiely and Riddiford, 1985), and of *D. melanogaster* (Wolfgang et al. 1986) that there are early and late cuticular proteins. The pattern of early and late synthesis is repeated in juvenoid-induced second pupae (Roberts and Willis, 1980c).

It is conceivable that once messages for cuticular proteins are present, the cell could control their sequential translation at the post-transcriptional level. Is this what is happening? There have been analyses of the products that come from *in vitro* translations of epidermal mRNA. This RNA has been stripped of its proteins so most sources of modifications that might alter the RNA *in vivo* have been eliminated. Studies examining translation products have been carried out with mRNAs taken from the epidermis of *M. sexta* (Riddiford, 1982) and *H. cecropia* (Binger, 1988). Products from messages of early cuticular proteins are found only early in the instar, and those for late proteins only late in the instar. During the period between apolysis and the onset of secretion of a new cuticle, the epidermis is engaged in making molting fluid to digest the old cuticle, and at this time mRNAs for cuticular proteins disappear.

The possibility remains that some non-proteinaceous modification has rendered mRNAs non-translatable. Riddiford's laboratory has carried out Northern analyses using probes for cuticular protein messages (Rebers and Riddiford, 1988; Horodyski and Riddiford, 1989). Once again messages for cuticular proteins are absent during the period when cuticular proteins are not being synthesized. The Northern analyses should detect any form of the message. Hence persistence of mRNA from molt to molt does not occur and cannot be the basis for juvenoid action. So it seems appropriate to reject mRNA stability and reusage as accounting for the anti-metamorphic action of juvenoids. One might only wish that the key evidence did not lie in so few experiments!

Accordingly attention has shifted to learning if juvenoids control metamorphosis at the level of the gene. The question is, what genes? There are two possibilities. The juvenoid receptor may be interacting directly with the genes coding for proteins that form the structures of each metamorphic stage. Alternatively, the juvenoid receptor may act on genes that code for regulatory proteins that in turn bind to or near the genes for structural proteins.

Direct Binding of JH and its Receptor to Cuticular Protein Genes

Juvenoids may work directly on the genes coding for the proteins that form the structures of each metamorphic stage, for example the cuticular proteins. How are we going to learn if juvenoids act at the level of the genes for such structural proteins? As papers in this volume by Riddiford and Wyatt illustrate, two paths of analysis are about to converge, studies on hormone receptors and analyses of cuticular proteins genes. Analyses of factors and genes unrelated to metamorphosis have made it possible to sort out the networks of regulatory proteins that control individual genes (see also papers in this volume by Scott and Suzuki).

Recent advances in molecular biology at last make it possible to learn whether a hormone interacts directly with a gene. Steroid hormones, thyroid hormones, and retinoic acid have receptors with similar structures (Evans, 1988). Each receptor is a complex protein with both DNA-binding and hormone-binding domains. Experiments where the DNA-binding domain of one receptor has been joined to the hormone-binding domain of another provide compelling evidence for the independence of these two components of the receptor molecule (Evans, 1988). A hormone binds to its hormone-binding domain and thereby exposes the DNA binding domain so that it can interact with a control element on a gene. Hormone-responsive genes in vertebrates have hormone-response-elements (HRE), located in close proximity to the coding region.

Sequences for HRE of a diverse array of vertebrate hormones are known. Once one has a receptor for a hormone and knows the sequence of its target gene (coding and regulatory regions), it is a routine matter to perform an assay to learn if a putative HRE taken from a gene of interest binds with appropriate specificity to the hormone-receptor complex. To perform such a "gel retardation" assay, one mixes a bit of naked DNA bearing the sequence of the HRE, with the hormone receptor complex and runs it out on a gel. If the complex is bound, the mobility of the DNA is reduced relative to its naked state.

Overlooking details and false starts, suppose the gene of a protein found in larval cuticle has a hormone-response element (HRE) that binds the JH receptor (JHR) complex. (Preliminary data in accord with this supposition can be found in Riddiford's paper in this volume). Does this mean that the JHR-HRE has anything to do with the "status quo" activity of juvenoids?

One interpretation of finding a HRE for the juvenoid receptor on a cuticular protein gene might be that we are seeing the site where juvenoids exert their "status quo" action, but there is an alternative explanation. Direct binding of the juvenoid receptor might only serve to control the RATE of cuticular protein production. We know that individual cuticular proteins show quite different rates and patterns of synthesis (Kiely and Riddiford, 1985). In an attempt to learn what signals influence these changes, Riddifords's group incubated epidermis *in vitro* under different hormonal conditions, and demonstrated that mRNA levels for a 14.6 kDa cuticular protein are suppressed by juvenoids, while the amount of mRNA for 12.8 and 13.3 kDa proteins increases (Riddiford et al. 1986).

Now the problem becomes how to differentiate between rate and "status quo" actions of the JHR-HRE? The standard techniques of measuring transcription rates under varying levels of juvenoids, carrying out gel retardation assays, and learning whether the HRE is part of an enhancer or promoter will not suffice. There is no obvious way information on hor-

monally-modified transcription rates can be used to distinguish between control of rate and "status quo" actions of juvenoids. Gel retardation assays only indicate that there is a short sequence of DNA associated with a coding region that is capable of recognizing a hormone-receptor complex. They do not reveal when binding of the HRE to the receptor occurs or even whether it occurs. It will not be a simple matter of learning whether the HRE is in an enhancer or a promoter, for both can be involved in tissue specificity and both can affect rates of synthesis. Indeed, the distinction between enhancers and promoters has become rather blurred (Dynan, 1989).

To demonstrate relevance for "status quo", we must establish that the JHR-HRE is occupied both when juvenoids are controlling a gene to keep it on and when they are controlling the same gene to keep it off. The actual distribution of two cuticular proteins from *H. cecropia* as shown in the Figure illustrates this requirement.

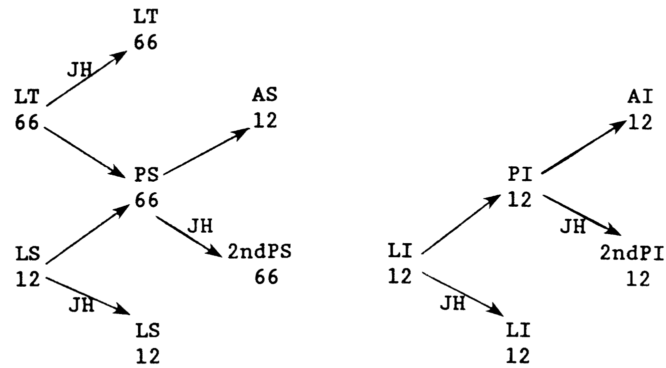


Figure 1. Distribution of two cuticular proteins in continuous populations of cells in different metamorphic stages of *Hyalophora cecropia*. Abbreviations: **A**, adult; **I**, intersegmental membrane; **JH**, juvenoids; **L**, larval; **P**, pupal; **S**, sclerite; **T**, tubercle; **12**, HCCP 12; ***66***, HCCP 66. (Data from Cox and Willis, 1985; Willis, 1981).

HCCP 12, one of the proteins illustrated in Fig. 1 is a major constituent of flexible cuticles. It is found in the larval and adult sclerites and in the intersegmental membrane of all stages (Cox and Willis, 1985). Direct sequencing of the N-terminus revealed that the first 43 amino acids are identical when the protein is extracted from either larval or adult integument (Willis, 1989). A probe based on the amino acid sequence was used to isolate a complete cDNA synthesized from mRNA taken from larval integument. Conceptual translation revealed an amino acid sequence that matches perfectly to the protein sequence (Binger and Willis, unpublished observations).

HCCP 66, the other protein shown in Fig. 1, and two other proteins closely related by sequence (HCCP 65 and HCCP 91) have been found only in rigid cuticles. Gross amino acid composition and N-terminal sequence data indicate that this group belongs to a distinct protein family (Willis, 1989). The N-terminal protein sequence for HCCP 66 is the same irrespective of whether the protein is isolated from larval tubercles, pupal sclerites, or pupal wings. To date, after a fairly extensive search, we have found only one major cuticular protein, HCCP 65, restricted to a single stage, pupal cuticle. This is a rather interesting protein for it shares 23 of its 25 N-terminal amino acids with HCCP 66 (Willis, 1989).

Any model we propose for the action of juvenile hormone must take into account the features shown in Fig. 1. In the same population of cells juvenoids may favor the synthesis of a particular protein in a particular cell at one stage and inhibit it at the next. This is the case for HCCP 12, a major protein in the larval integument, both sclerite and intersegmental membrane. In the pupa HCCP 12 is restricted to the intersegmental membrane. When the epidermis of the pupal sclerite undergoes metamorphosis in the absence of juvenoids, the cells make and secrete HCCP 12 once again. Thus, for HCCP 12, the "status quo" hypothesis calls for resynthesis in the supernumerary larval sclerite, but for prevention of synthesis in the sclerite of the second pupa. In the adjacent intersegmental membrane, HCCP 12 is synthesized irrespective of whether the molt is in the presence or absence of

juvenoids. For HCCP 66, the "status quo" condition requires resynthesis in the tubercle and prevention of synthesis in the sclerite epidermis surrounding each tubercle.

Fig. 1 makes it possible to design a critical test for the relevance of binding of the juvenoid receptor (JHR) to a juvenoid response element (HRE) of a cuticular protein gene. If we were interested in protein 12 in the sclerite, we would expect JHR to be bound to the gene in the critical period for a supernumerary larval molt, when the anti-metamorphic action of juvenoids is to keep the gene on, and again during the critical period for formation of a second pupa when the anti-metamorphic action of juvenoids is to keep the gene off. If the HRE is not occupied during both of these critical periods, then we would have reason to doubt that juvenoids exert their "status quo" action by binding to genes for structural proteins.

To learn when the hormone receptor complex is bound to its response element, one has to resort to a technique called "*in vivo* footprinting", a technique that minimizes the possibility that proteins will shift on the chromosomes during experimental manipulation (Ephrussi et al. 1985; Becker and Schütz, 1989). To accomplish this one adds dimethyl sulfate (DMS) to intact cells. DMS is capable of methylating guanine residues, but its ability to do this is altered if proteins are bound to the DNA. One can compare DNA from cells that are in different physiological states when they were exposed to DMS and determine what particular sites were protected. The DNA is cleaved after DMS treatment, run out on a sequencing gel, blotted and identified with a probe specific for the gene of interest.

In theory then, we should be able to learn if the HRE is protected during critical periods for juvenoid action. Or, alternatively, if this binding is only during periods when juvenoids may be regulating the rate of transcription for the particular cuticular protein gene. We most likely will have found the site of the "status quo" action of juvenoids, if we find that the JH-receptor complex is bound to the HRE of cuticular protein genes during all critical periods for juvenoid action, both those critical periods where "status quo" action is to keep a gene on and other periods where its action is to keep the same gene off. But suppose this test fails. Suppose the JH-Receptor complex fails to bind during all critical periods for "status quo" action. Where are we?

Indirect Action on Cuticular Protein Genes

There is another way juvenoids might be exerting their "status quo" action and that is at the level of chromatin (Willis, 1981). If juvenoids were to act at this level, their action would be more generic than a direct action on genes for structural proteins.

Chromatin, DNA complexed to proteins (both histones and non-histone chromosomal proteins) exists in several forms, clearly described by Van Holde (1989). Briefly, chromatin may be found in a tightly coiled configuration where regulatory proteins have no access to genes. Alternatively, regions of chromatin may be "open", forming open-looped domains where regulatory proteins can interact with cis-acting regulatory elements. Whether or not a particular gene in an open domain is being transcribed depends on whether trans-acting regulatory proteins are present.

Hence, for a gene to be active, regulatory proteins must be associated with it. This requirement reveals three other levels at which juvenoids might act to enforce the "status quo": opening of chromatin domains, synthesis of regulatory factors, and ability of regulatory factors to translocate from cytoplasm to nucleus.

The first possibility is that juvenoids might prevent the opening of chromatin domains, hence regulatory factors would have no place to bind. This would be a very effective way for maintaining the "status quo", for it would not interfere with repetition of the series of syntheses that characterized the activities of the epidermis during the previous molt cycle.

Open chromatin domains can be recognized because the entire region is sensitive to DNase I. Such moderately DNase I sensitive regions can extend over 100 kb. How do open domains arise? There is evidence that topoisomerase II plays a role in opening up a domain. If it does, it must recognize some other chromosomal proteins that indicate where it should act. Acetylation and ubiquination of histones have also been recognized as important in the opening of chromatin (Van Holde, 1989). Therefore, inhibitors of any of these activities might be mimics of juvenoids. Novobiocin, among other actions, works as an inhibitor of topoisomerase II, and I have tested it on *Oncopeltus fasciatus* larvae and *T. molitor* pupae. Two doses are routinely used with vertebrate cells, 1 mg/ml and 500 µg/ml. *O. fasciatus*

nymphs injected with doses to obtain either final concentrations (on a per gram basis) molted into perfectly normal adults. The higher dose caused *T. molitor* some trouble with ecdysis, but there appeared to have been good pharate adult development. The lower dose yielded all normal adults. Such simple experiments, where inhibitors are used on whole animals, produce negative results that are not interpretable, for without further tests we do not know if the drug even reaches the intended targets. So while the experiment was simple and worth trying, the negative results can not be taken to eliminate the possible involvement of topoisomerase II in regulating metamorphosis.

The second possibility, that juvenoids might block the synthesis of new regulatory proteins, would move juvenoid inhibition to another set of genes, those for regulatory proteins. At present there is no information on this possibility, but reference to Fig. 1 shows how complicated this might become, for adjacent cells would need different juvenoid-sensitive factors.

The third possibility is that juvenoids might prevent the translocation of trans-acting regulatory factors into the nucleus. There is precedent for such regulation. The synthesis of the κ-light chain element in mammalian B cells is regulated, in part, by a protein called NF-κB. Early experiments indicated that this factor was present only in the nucleus of mature B lymphocytes. It was soon learned that the factor was fairly ubiquitous, found in the cytoplasm of diverse cell types, but in the cytoplasm it is complexed with an inhibitor (see Lenardo and Baltimore, 1989 for review). When appropriate stimuli come to the cell, the inhibitor is phosphorylated, and NF-κB is translocated into the nucleus. This phosphorylation is part of the protein kinase C cascade. Movement into the nucleus can be stimulated with phorbol esters, known stimulators of protein kinase C.

Could juvenoids be involved in the phosphorylation of a regulatory complex comparable to NF-κB? Could juvenoids act by preventing positive regulatory molecules from moving into the nucleus or alternatively by facilitating the translocation of negative regulatory molecules? Is there any precedent for suggesting that juvenoids act via the protein kinase C cascade? Two relevant papers have been published recently on this subject. Calcium and protein kinase C have been implicated in the action of juvenoids in male accessory glands of *D. melanogaster* (Yamamoto et al. 1988). The best characterized action of juvenoids is to increase patency (spaces between follicle cells) in the ovary of *R. prolixus*. This rapid action of juvenoids involves an increase in the activity of the membrane Na^+-K^+ ATPase. Sevala and Davey (1989) have placed protein kinase C in the short link between juvenoids and the activation of the Na^+-K^+ ATPase.

I have injected reasonable doses (0.1-1 μM, final concentration) of two phorbols, TPA and the more soluble phorbol 12, 13 dibutyrate, into both *O. fasciatus* and *T. molitor*. They do not cause any disturbance in development. In some systems their action requires a calcium ionophore, so, in very preliminary experiments, I have also presented phorbols (1 μM) mixed with the standard ionophore A23187 (500 nM final concentration). Once again no effect on metamorphosis was observed. The same caveats as discussed with the experiments on novobiocin apply here. It would be premature to rule out any hypothesis based on the failure to find an effect of a drug in a whole animal.

SUMMARY

It is unlikely that juvenoids act by inhibiting mRNA synthesis and forcing the reutilization of old messages. Rather they must be effecting the "status quo" by favoring the resynthesis of "old" messages and preventing the synthesis of new ones. Finding that the juvenoid receptor binds to a gene for a protein used during a particular metamorphic stage will not illuminate whether the binding is significant for the "status quo" action of juvenoids or whether it is merely an indication that juvenoids regulate the rate of transcription of that gene during the intermolt period. To differentiate between these two possibilities, we must apply new methods. That juvenoids might act by effecting a generic action on chromatin to keep domains frozen at their present state is certainly tenable and testable. It is also possible that juvenoids work to prevent the synthesis of new regulatory factors, factors that act on the genes for the structural proteins for the next metamorphic stage. Finally, it is possible that we may find that juvenoids exert their "status quo" action via a membrane receptor and the protein kinase C cascade, and thereby affect the translocation of regulatory factors into the nucleus. If this last scenario were to be correct, we would be back to a model comparable to that presented by Wigglesworth a half century ago, with juvenoids affecting enzymes in the cytoplasm.

ACKNOWLEDGMENTS

Research was supported by the National Science Foundation NSF DCB 8607865. I am grateful to Gene E. Robinson and John S. Willis for their constructive criticisms of the manuscript.

This paper is dedicated to Professor Vincent B. Wigglesworth in honor of his 90th birthday and his germinal insights into action of juvenoids in regulating metamorphosis.

REFERENCES

Andersen, S. O., Chase, A. M., and Willis, J. H. 1973. The amino-acid composition of cuticles from *Tenebrio molitor* with special reference to the action of juvenile hormone. Insect Biochem. 3: 171-180.

Becker, P. B., and Schütz, G. 1988. Genomic Footprinting. Gen. Engin.. Prin. Meth. 10: 1-19.

Binger, L. C. 1988. Translation of epidermal RNA from different anatomical regions and metamorphic stages of the giant silkmoth *Hyalophora cecropia* using rabbit reticulocytes and wheat germ preparations. Master's Thesis. University of Illinois, Urbana, Champaign.

Chase, A. M. 1967. Effects of actinomycin D on development in pupae of *Tenebrio molitor*. Nature 215: 1516-1517.

Chase, A. M. 1970. Effects of antibiotics on epidermal metamorphosis and nucleic acid synthesis in *Tenebrio molitor*. J. Insect Physiol. 16: 865-884.

Cox, D. L., and Willis, J. H. 1985. The cuticular proteins of *Hyalophora cecropia* from different anatomical regions and metamorphic stages. Insect Biochem. 15: 349-362.

Dynan, W. S. 1989. Modularity in promoters and enhancers. Cell 58: 1-4.

Ephrussi, A., Church, G. M., Tonegawa, S., and Gilbert, W. 1985. B lineage-specific interactions of an immunoglobulin enhancer with cellular factors in vivo. Science 227: 134-140.

Evans, R. M. 1988. The steroid and thyroid hormone receptor superfamily. Science 240: 889-895.

Henikoff, S., and Eghtedarzadeh, M. K. 1987. Conserved arrangement of nested genes at the Drosophila *Gart* locus. Genetics 117: 711-725.

Horodyski, F. M., and Riddiford, L. M. 1989. Expression and hormonal control of a new larval cuticular multigene family at the onset of metamorphosis of the tobacco hornworm. Devel. Biol. 132: 292-303.

Kiely, M. L., and Riddiford, L. M. 1985. Temporal programming of epidermal cell protein synthesis during the larval-pupal transformation of *Manduca sexta*. Roux's Arch. Dev. Biol. 194: 325-335.

Lawrence, P. A. 1969. Cellular differentiation and pattern formation during metamorphosis of the milkweed bug *Oncopeltus*. Devel. Biol. 19: 12-40.

Lenardo, M. J., and Baltimore, D. 1989. NF-κB: A pleiotropic mediator of inducible and tissue-specific gene control. Cell 58: 227-229.

Phillips, D. R., and Loughton, B. G. 1979. The effect of inhibition of RNA and protein synthesis on the development of larvae of *Locusta migratoria*. Insect Biochem. 9: 241-245.

Rebers, J. F., and Riddiford, L. M. 1988. Structure and expression of a *Manduca sexta* larval cuticle gene homologous to *Drosophila* cuticle genes. J. Mol. Biol. 203: 411-423.

Riddiford, L. M. 1982. Changes in translatable mRNAs during the larval-pupal transformation of the epidermis of the tobacco hornworm. Devel. Biol. 92: 330-342.

Riddiford, L. M. 1987. Hormonal control of sequential gene expression in insect epidermis. UCLA Symp. Mol. Cell. Biol. 49: 211-222.

Riddiford, L. M., Baeckmann, A., Hice, R. H., and Rebers, J. 1986. Developmental expression of three genes for larval cuticular proteins of the tobacco hornworm, *Manduca sexta*. Develop. Biol. 118: 82-94.

Roberts, P. E., and Willis, J. H. 1980a. The cuticular proteins of *Tenebrio molitor*. I. Electrophoretic banding patterns during postembryonic development. Devel. Biol. 75: 59-69.

Roberts, P. E., and Willis, J. H. 1980b The cuticular proteins of *Tenebrio molitor*. II. Patterns of synthesis during postembryonic development. Devel. Biol. 75: 70-77.
Roberts, P. E., and Willis, J. H. 1980c. Effects of juvnile hormone, ecdysterone, actinomycin D, and mitomycin C on the cuticular proteins of *Tenebrio molitor*. J. Embryol. Exp. Morph. 56: 107-123.
Sevala, V. L., and Davey, K. G. 1989. Action of juvenile hormone on the follicle cells of *Rhodnius prolixus*: Evidence for a novel regulatory mechanism involving protein kinase C. Experientia_ 45: 355-356.
Shirras, A. D., and Bownes, M. 1989. cricklet: a locus regulating a number of adult functions of *Drosophila melanogaster*. Proc. Natl. Acad. Sci. 86: 4559-4563.
Socha, R., and Sehnal, F. 1972. Inhibition of adult development in *Tenebrio molitor* by insect hormones and antibiotics. J. Insect Physiol. 18: 317-337.
Van Holde, K. E. 1989. "Chromatin." Springer-Verlag, New York,.
Wielgus, J. J. 1983. Stimulation of intermoult cuticle deposition by a haemolymph trophic factor in the tobacco hornworm, *Manduca sexta*. Insect Biochem. 13: 313-322.
Wigglesworth, V. B. 1940. The determination of characters at metamorphosis in *Rhodnius prolixus* (Hemiptera). J. Exp. Biol. 17: 201-222.
Wigglesworth, V. B. 1959. Metamorphosis, polymorphism, differentiation. Sci. Amer. 200 #2: 100-106.
Wigglesworth, V. B. 1970. "Insect Hormones." Oliver & Boyd, Edinburgh.
Williams, C. M., and Kafatos, F. C. 1971. Theoretical aspects of the action of juvenile hormone. Mitt. Schweiz. Entomol. Ges. 44: 151-162.
Willis, J. H. 1981. Juvenile hormone: the status of "status quo." Amer. Zool. 21: 763-773.
Willis, J. H. 1986. The paradigm of stage-specific gene sets in insect metamorphosis: time for revision!. Arch. Insect Biochem. Physiol. Suppl. 1: 47-57.
Willis, J. H. 1989. Partial amino acid sequences of cuticular proteins from *Hyalophora cecropia*. Insect Biochem. 19: 41-46.
Willis, J. H. 1990. The epidermis and metamorphosis. in: "The Physiology of Insect Epidermis." A. Retnakaran and K. Binnington, Eds. Inkata Press, Victoria. in press.
Willis, J. H., and Hollowell, M. P. 1976. The interaction of juvenile hormone and ecdysone: antagonistic, synergistic, or permissive? pp. 270-287 in: "The Juvenile Hormones." L. I. Gilbert, Ed. Plenum Press, New York.
Willis, J. H., Rezaur, R., and Sehnal, F. 1982. Juvenoids cause some insects to form composite cuticles. J. Embryol. Exp. Morph. 71: 25-40.
Wolfgang, W. J., Fristrom, D., and Fristrom, J. W. 1986. The pupal cuticle of *Drosophila:* differential ultrastructural immunolocalization of cuticle proteins. J. Cell Biol. 102: 306-311.
Yamamoto, K., Chadarevian, A., and Pellegrini, M. 1988. Juvenile hormone action mediated in male accessory glands of *Drosophila* by calcium and kinase C. Science 239: 916-919.

THE INTEGRATION OF THE GENOME OF A SEGMENTED DNA VIRUS IN THE HOST INSECT'S GENOME

Jo-Ann G. W. Fleming and Max D. Summers

Department of Entomology and Institute of Biosciences and Technology
Texas A & M University
College Station, Texas

INTRODUCTION

Polydnaviruses have a unique relationship with the parasitoids in which they occur naturally. This virus-insect association is novel not only in being constitutive but also in being mutualistic rather than pathogenic. A major component of the normal microflora of the wasps' oviducts, polydnaviruses appear to play an essential role in the reproductive success of the parasitoids and hence to their survival as a species.

Insects like other organisms possess efficient, albeit comparatively poorly understood, immune mechanisms by which they are protected against invasion by microorganisms (phagocytosis, nodulation) or other invertebrates (encapsulation). During the course of coevolution obligate endoparasites like parasitoids clearly have developed mechanisms by which they are able to overcome or circumvent the immune defenses of their insect hosts, thereby ensuring their normal growth, development and survival. Polydnaviruses appear to be one mechanism by which several species of ichneumonid and braconid parasitoids effect the immunocompromise of their lepidopteran hosts such that their eggs and endoparasitc larval stages are not recognized as foreign and/or are not effectively encapsulated by their host's hemocytes (Edson et al., 1981; Stoltz and Vinson, 1979b). Transferred from the wasps' oviducts to the parasitized hosts in the course of normal oviposition, polydnaviruses rapidly infect host tissues (Stoltz and Vinson, 1979a). In the *Campoletis sonorensis-C. sonorensis* virus (CsV) system, it has been demonstrated that viral gene expression occurs within 2 hr after oviposition and continues throughout the period of endoparasitic wasp development (Blissard et al., 1986; Fleming et al., 1983). Although the functions of the various viral gene products expressed in the parasitized host in suppressing of the host immune response and in dramatically altering the host's physiological status are not known, injection studies strongly suggest that the immunosuppression and a significant number of the other pronounced changes observed in parasitized larvae are a consequence of viral gene expression, since purified virus alone induces such changes and treatments like UV irradiation or psoralen exposure of the virus abolish its effect (Edson et al., 1981; Theilmann and Summers, 1987). *In vivo* protection studies support the essential nature of these virally induced changes in the outcome of the host-parasitoid interaction: washed, virus-free eggs fail to develop in host larvae after manual implantation but do complete normal development if purified virus is co-injected (Edson et al., 1981). Thus, although a detailed discussion of the role of polydna-viruses in abrogating the immunity of parasitized hosts is beyond the scope of this paper, polydnaviruses clearly are an integral part of the life cycle of certain parasitoid species.

In view of the importance of polydnaviruses to the reproductive success of certain wasp species, it is not surprising that all females in species in which polydnaviruses have been detected appear to contain these viruses in their oviducts (Stoltz and Vinson, 1979b). Moreover, available evidence from comparative studies on the DNA electrophoretic profiles of polydnaviruses from several braconid and ichneumonid species and from cross protection

Molecular Insect Science, Edited by
H. H. Hagedorn *et al.*, Plenum Press, New York, 1990

experiments suggest that polydnaviruses are species specific, in terms of both the wasp species in which given polydnaviruses are found and replicate normally and in the lepidopteran host species in which they elicit altered physiological responses (Jones and Sreekrishna, 1986; Krell and Stoltz, 1979; Krell and Stoltz, 1980; Krell et al., 1982; Vinson, 1977). These observations imply that these symbiotic viruses are transmitted between wasp generations by a mechanism that both is highly efficient and maintains the species specificity of the virus-wasp association even in cases where the wasps develop in hosts parasitized by more than one parasitoid species. Vertical transmission fulfills these criteria.

Very limited information on the genetics of these viruses is available. Inheritance studies on *Cotesia melanoscela* virus (CmV) found in the braconid *C. melanoscela* suggest that CmV is vertically transmitted, but the molecular mechanism by which this is accomplished was not clearly indicated (Stoltz et al., 1986). Our work on the ichneumonid *C. sonorensis* and its polydnavirus CsV indicates that the genetic relationship between the virus and the parasitoid is a close one. We have directed our efforts to an examination of the physical basis of that relationship.

The CsV Genome and Occurrence of CsV DNAs in Wasps

Polydnaviruses historically have been detected in wasps by electron microscopy as dense particulate secretions in the oviducts and have been shown to replicate primarily in the calyx epithelial cells of the oviduct (Norton and Vinson, 1983; Norton et al., 1975). The first evidence that polydnaviral DNA is not restricted to these tissues was our demonstration that all body segments (head, thorax, and abdomen) of both male and female *C. sonorensis* wasps contain CsV specific DNA that persists throughout the life of the animals (Fleming and Summers, 1986). Morevoer, except for the female abdomena which contain the oviducts, the tissues of male and female wasps contain approximately equivalent, picogram amounts of the viral DNA. Because the levels appeared similar in the nonoviduct tissues from both sexes and virus particles have not been observed in male wasps, males are used as a convenient source of chromosomal DNA for the physical analyses since it is unnecessary to remove the viruliferous oviducts.

Analysis of the virus specific DNA in males indicated that two differently organized forms exist in somatic tissue. The predominant form is detectable as offsize restriction fragments when compared to similarly digested viral DNA extracted from purified virions from oviducts (Fleming and Summers, 1986). The form yielding offsize restriction fragments also is present in the cellular DNA isolated from heads and thoraces of females. This was the first indication that polydnavirus DNAs might be integrated, but the genetic complexity of the viral genome suggested alternative explanations for the offsize restriction fragments, necessitating our more detailed analyses discussed below. A second form that is identical to the superhelical DNAs found encapsidated in virions also is found in male tissues. On the basis of the relative intensities of the offsize and virus-like forms in autoradiographs as well as on the basis of the amount of typical virus-like CsV specific DNA isolated from over a hundred males, the virus-like form clearly is an extremely minor component of the total polydnavirus specific DNA in males (Fleming and Summers, 1986). The detection of even trace amounts of virus-like CsV DNA in males was unexpected, since the apparent restriction of high levels of viral replication to mature oviducts suggests hormonal regulation. However, although our work has focused on the predominant form that is unlike the virion-derived viral DNA, this minor, virus-like form should also be considered in future studies investigating the replication strategy of polydnaviruses. The circular DNAs found may indicate that low levels of virus replication may occur in certain male tissues, which is of interest from the standpoint of the regulation of viral and/or host genes involved in this process. A small portion of the viral DNA in somatic cells may persist as episomes. This episomal form could not only be transmitted through the gametes but also could be preferentially used as the templates for replication even if it is quantitatively minor. It is of interest that the blot analysis of the one CmV DNA examined in *C. melanoscela* was consistent with that DNA being episomal in that braconid species (Stoltz et al, 1986). It is not clear whether the apparent lack of virus specific offsize restriction fragments in that braconid species results from technical differences, the extremely limited extent of the analysis of the braconid, or the significant taxonomic differences between CsV and CmV, which are classified in subfamilies A and B, respectively, of the Polydnaviridae (Brown, 1986;Stoltz et al., 1984).

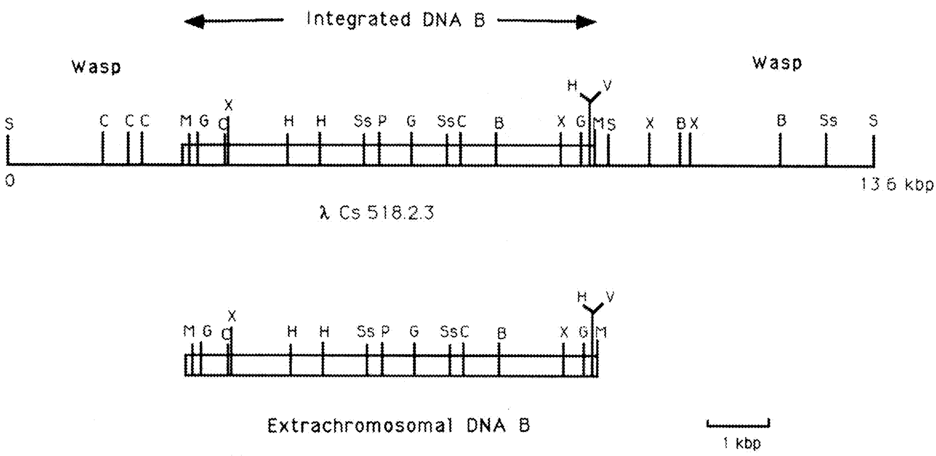
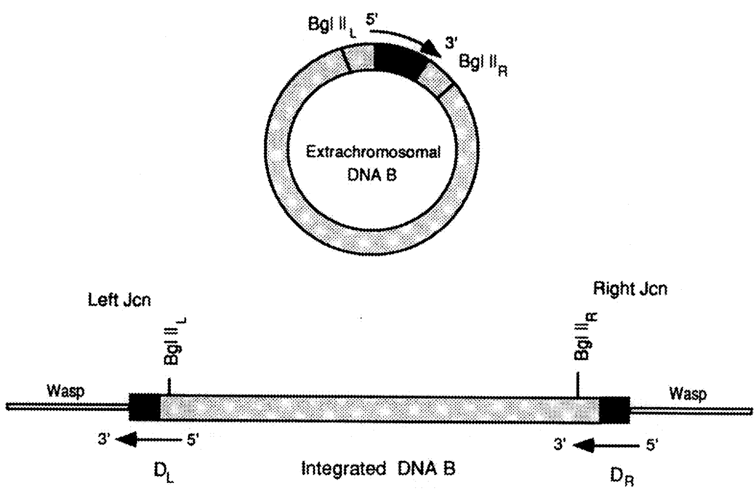


Fig. 1. Physical maps of extrachromosomal and integrated CsV DNA B. Physical maps of (linearized) extrachromosomal viral DNA B and a lambda-wasp recombinant containing viral DNA B-specific sequences were determined by restriction endonuclease digestion and reciprocal Southern blot hybridizations. The DNA B-specific sequences in (cloned) wasp and extrachromosonal DNA are colinear.



Left Repeat 5' CCCGGGTGCTTATCTGTTCCCGCAGGAAGCGTACATCGTGT 3'
Extrachromosomal 5' CCCGGGTGCTTATCTGTTCCCGCAGGAAGCGTACATCGTGT 3'
Right Repeat 5' CCCGGGAGATCATCTGTTCCCGAAGCAAGCGTATATCGTGT 3'

Fig. 2. Direct repeat sequences in viral DNA B-specific DNA. The nucleotide sequences of the junctions of integrated CsV DNA B and the comparable *Bgl*II fragment of extrachromosomal DNA B indicate that imperfect direct repeats occur at the termini of integrated DNA B, but a single copy occurs in extrachromosomal DNA B, which is pictured schematically as a covalently closed circular DNA as it exists in virions. The sequences of the direct repeat at each junction and in extrachromosomal viral DNA B are given below the schematic diagram indicating the location and orientations of the sequences as they occur in DNA B-specific wasp and encapsidated viral DNAs. Nucleotides that differ in the direct repeat at the right junction and the copy in extrachromosomal DNA are underlined.

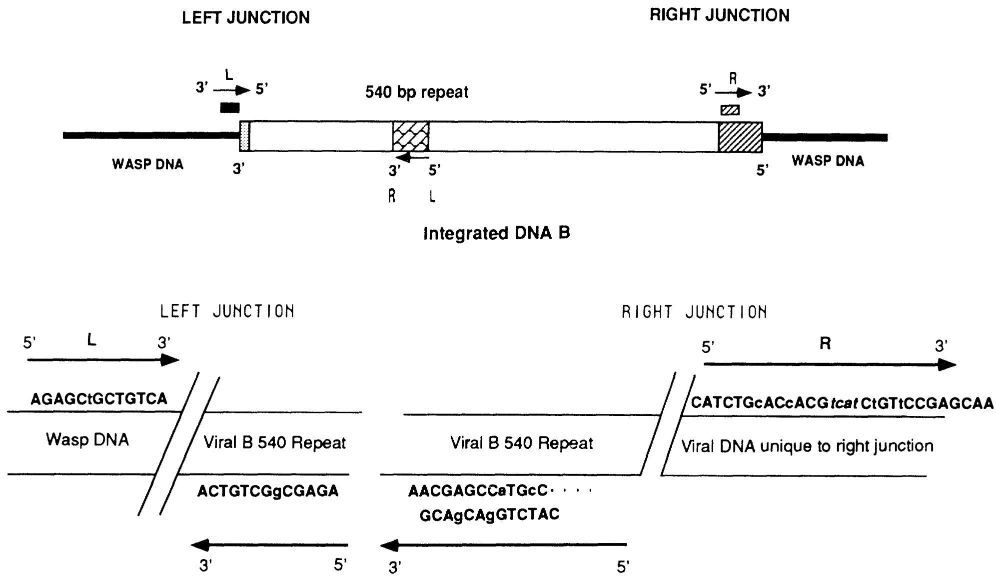


Fig. 3. Related junction-associated and 540 bp repeat element sequences. The nucleotide sequences of wasp and viral DNAs at or near the junctions of integrated CsV DNA B and wasp DNA and the published sequence of the 540 bp repeat element on extrachromosomal DNA B (see Theilmann and Summers, 1987) were compared. Wasp sequences at the left junction and viral sequences near the right junction are related to sequences near the 5' and 3' ends of the 540 bp repeat element on DNA B, respectively, and constitute imperfect inverted repeats with the 540 bp repeat element sequences. The location and orientations of the related sequences are shown schematically.

By analogy with other integrated viruses detected in many eucaryotic species, the CsV-specific offsize restriction fragments in *C. sonorensis* cellular DNA suggested that the CsV DNAs initially investigated are integrated. Due to the physical and genetic complexity of the CsV genome, other possible explanations for the differently organized viral DNA seemed plausible. Like other polydnaviruses, CsV has a large segmented genome of superhelical DNAs (Brown, 1986; Krell, et al., 1982). The 28 circular CsV DNAs, which range in size from 6.0 to 21 kBp, exhibit complex patterns of cross hybridization (Blissard, et al., 1986; Blissard et al., 1989; Blissard et al., 1986; Fleming and Summers, 1986; Theilmann and Summers, 1987). These patterns indicate that CsV is not a defective virus, but during evolution its genome has undergone multiple duplication, rearrangement and/or recombinational events. At least two gene families of unknown function exist in the genome: (i) one contains two genes located on superhelical DNA W (and possibly also on DNAs C and M) which are highly expressed in parasitized host larvae, and (ii) the second family is composed of 540 bp repeat elements that exist singly or in tandem arrays on most of the viral DNA segments and are expressed in either the wasp, the parasitized host, or both, depending on the gene examined (Blissard et al., 1989; Theilmann and Summers, 1987; Theilmann and Summers, 1988). Although the limited sequence similarity but very similar temporal and species specificity of expression of the two genes on DNA W might be consistent with convergent evolution as well as with duplication followed by significant gene divergence, the extensive distribution of the 540 bp repeat element genes within the CsV genome and the species specific differences in transcription of the various members are not easily accorded with convergent evolution and are stronger evidence for gene duplications or DNA rearrangements during polydnavirus evolution. Furthermore, these widely distributed but imperfectly conserved elements could theoretically facilitate intra-genomic recombination. Thus, even though the genome of the polydnavirus isolated from our highly inbred *C. sonorensis* colony appears to be stable and CsV has received substantially more attention than other polydnaviruses, our still comparatively rudimentary understanding of the complex CsV genome did not allow us to dismiss the possibility that

the offsize restriction fragments represented products of recombination that, for an unknown reason, were not efficiently packaged in virus particles and/or occurred more frequently in nonovarian tissues.

CsV DNAs Are Integrated

To distinguish between these two hypothetical explanations for the differently organized viral DNA in nonovarian tissues, we constructed a *C. sonorensis* genomic library from male wasp DNA and screened it with viral DNA probes. The probes used represent either superhelices that have been cloned in their entirety (B, H, M, O' and W[6.7, 8.5, 10.4, 11.2, and 15.8 kBp, respectively]) or represent significant cloned portions of them (DNA Q[12.2 kbp]) (Blissard et al., 1987; Fleming and Summers, 1986; Theilmann and Summers, 1987). Based on the hybridization of these viral DNAs with total CsV DNA isolated from virus from the oviducts, these six DNAs are related to varying extents, but DNAs Q and W do not appear to be related at levels detectable under the stringency conditions used (Blissard et al., 1989; Fleming and Summers, 1986). Although these initial analyses focused primarily on CsV superhelical DNA B (6.7 kbp), a limited number of offsize restriction fragments are detectable with each of the viral DNA probes (Fleming and Summers, 1986; and unpub-lished observations). In each case, the viral sequences that are altered in wasp cellular DNA can be reproducibly mapped to discrete regions of the given superhelical DNAs, even though cellular DNA isolated from several thousand wasps representing over 50 generations was used for the mapping. For example, this more detailed mapping indicated that only a 415 bp Bgl II fragment of CsV superhelical DNA B appears to be altered in wasp chromosomal DNA (Fleming and Summers, unpublished observations). It should be noted, however, that our *C. sonoresis* colony is highly inbred, and the apparent level of stability within the species may therefore be somewhat exaggerated.

Using these data as background information, the recombinant lambda clones homologous to cloned CsV DNA B (pBE6700) were physically mapped by standard restriction endonuclease digestion and reciprocal Southern blot hybridization. In cloned wasp DNA the viral DNA B specific sequences are colinear with virion-derived CsV DNA B and are flanked by sequences that do not hybridize with pBE6700 (Fig. 1). Portions of the 415 bp *Bgl*II fragment to which the offsize restriction fragments map occur at the junctions of the DNA B specific and the unrelated flanking sequences. To determine the viral or nonviral origin of the flanking sequences, a series of reciprocal Southern blot hybridizations using the entire CsV genome, pBE6700 and the flanking sequences subcloned from the left and right junctions were done. In summary, these experiments, which are reported more fully elsewhere (Fleming and Summers, unpublished observatins), showed that the flanking sequences did not hybridize with CsV DNA under low stringency conditions to levels detectable in long autoradiographic exposures. The flanking sequences, however, did hybridize to a limited number of fragments in digested male wasp DNA under high stringency conditions. Thus, CsV DNA B is covalently linked to sequences of nonviral, i. e. wasp, origin and is integrated without significant rearrangement. Because all of the viral DNAs that have been examined detect offsize restriction fragments in *C. sonorensis* cellular DNA regardless of their apparent relatedness to DNA B, these DNAs also likely are stably integrated in unique or low copy number wasp DNA, and we postulate that all of the viral DNAs in the CsV genome are integrated. The polydnavirus CsV therefore appears to be the first documented integrated, nonretroviral DNA virus of insects.

Nucleotide sequence analysis of the viral specific DNA at the left and right junctions, the flanking sequences and the homologous 415 bp Bgl II fragment from extrachromosomal, i.e. virion-derived, DNA B was done to compare the structural organization and sequence similarity of the various DNAs (Fleming and Summers, manuscript submitted for publication). Comparatively short imperfect direct repeats occur at both termini of integrated DNA B, but a single copy is found in the extrachromosomal superhelical viral DNA B (Fig. 2). The absence of internal inverted repeats and target sites duplications suggest that integrated CsV DNA B is not closely related to retrotransposons or other characterized transposable elements. It presently is not known whether this organizational pattern is conserved on the other superhelical DNAs in the genome. The importance of the observed pattern for polydnavirus replication therefore is not known. Our continuing studies on CsV DNA integration should help to resolve these questions.

Because repeat elements frequently are involved in replication of other eucaryotic viruses and because the members of the 540 bp repeat element gene family appear to exist on most superhelices in the CsV genome, the sequence similarity of the viral and wasp DNAs at or near the junctions and the single 540 bp repeat element on DNA B was investigated (Theilmann and Summers, 1988). Sequences at or near the left and right junctions are related to sequences near either end of the 540 bp repeat element gene located internally in integrated DNA B and appear to form imperfect inverted repeats (Fig. 3). Whether this sequence relatedness is functionally significant is not clear at this time, although a possible role in the stabilization of conformations that may be important to polydnaviral replication can be envisioned.

The integration of CsV DNA B and the highly probable integration of the other DNAs in the viral genome are consistent with CsV being maintained in *C. sonorensis* as a provirus. Whether the integrated viral DNA acts as the template for replication is not clear, especially in view of the detection of limited amounts of extrachromosomal viral DNA in males. Our continuing molecular genetic analyses of the replication strategy of polydnaviruses should resolve this point and clarify the relationship of CsV DNAs with other mobile genetic elements and their possible utility in the genetic manipulation of insects. Regardless of which type of viral DNA ultimately is shown to act as template for viral DNA replication, vertical transmission of CsV and other polydnaviruses is extremely likely. Our results extend the characterization of an unusual symbiotic relationship between a virus and an insect to a molecular level. The evidence for a genetic relationship between an endoparasitic insect and a polydnavirus by which the parasite transfers genetic information that results in the physiological and behavioral modification of another invertebrate to the ultimate selective advantage of both the endoparasite and the virus is another example of the novel but exquisite mutualisms that have evolved in the insect world.

REFERENCES

Blissard, G.W., Fleming, J.G.W., Vinson, S.B. and Summers, M.D. 1986. *Campoletis sonorensis* virus: Expression in *Heliothis virescens* and identification of expressed sequences. J. Insect Physiol. 32: 351-359.

Blissard, G.W., Smith, O.P. and Summers, M.D. 1987. Two related viral genes are located on a single superhelical DNA segment of the multipartite *Campoletis sonerensis* virus genome. Virol. 160: 120-134.

Blissard, G.W., Theilmann, D.A. and Summers, M.D. 1989. Segment W of *Campoletis sonorensis* virus: Expression, gene products, and organization. Virol. 169: 78-89.

Blissard, G.W., Vinson, S.B. and Summers, M.D. 1986. Identification, mapping, and *in vitro* translation of *Campoletis sonorensis* virus mRNAs from parasitized *Heliothis virescens* larvae. J. Virol. 57:318-327.

Brown, F. 1986. The classification and nomenclature of viruses. Intervirol. 25: 141-143.

Edson, K.M., Vinson, S.B., Stoltz, D.B. and Summers, M. D. 1981. Virus in a parasitoid wasp: suppression of the cellular immune response in the parasitoid's host. Science 211: 582-582.

Fleming, J.G.W., Blissard, G.W., Summers, M.D. and Vinson, S.B. 1983. Expression of *Campoletis sonorensis* virus in the parasitized host, *Heliothis virescens*. J. Virol. 48: 74-78.

Fleming, J.G.W. and M. D. Summers, M.D. 1986. *Campoletis sonorensis* endoparasitic wasps contain forms of *C. sonorensis* virus DNA suggestive of integrated and extrachromosomal polydnavirus DNAs. J. Virol. 57: 552-562.

Jones, D. and Sreekrishna, S. 1986. Comparison of viral ultrastructure and DNA banding patterns from the reproductive tracts of eastern and western hemisphere Chelonus spp. J. Invert. Pathol. 47: 105-115.

Krell, P.J. and Stoltz, D.B.. 1979. Unusual baculovirus of the parasitoid wasp *Apanteles melanoscelus*: isolation and preliminary characterization. J. Virol. 29: 1118-1130.

Krell, P.J. and Stoltz, D.B. 1980. Virus-like particles in the ovary of an ichneumonid wasp: purification and preliminary characterization. Virol. 101: 408-418.

Krell, P.J. Summers, M.D. and Vinson, S.B. 1982. Virus with a multipartite superhelical DNA genome from the ichneumonid parasitoid, *Campoletis sonorensis*. J. Virol. 43: 859-870.

Norton, W.N. and Vinson, S.B. 1983. Correlating the initiation of virus replication with a specific pupal developmental phase of an ichneumonid parasitoid. Cell Tissue Res. 231: 387-398.

Norton, W.N., Vinson, S.B. and Stoltz, D.B. 1975. Nuclear secretory particles associated with the calyx cells of the ichneumonid parasitoid *Campoletis sonorensis*. Cell Tissue Res. 162: 195-208.

Stoltz, D.B. 1981. Polydisperse viral DNAs in ichneumonid ovaries: A survey. Can. J. Microbiol. 27: 123-131.

Stoltz, D.B., Guzo, D. and Cook., D. 1986. Studies on polydnavirus transmission. Virol. 155: 120-131.

Stoltz, D.B., Krell, P.J., Summers, M.D. and Vinson, S.B. 1984. Polydnaviridae-a proposed family of insect viruses with segmented, double-stranded, circular DNA genomes. Intervirol. 21: 1-4.

Stoltz, D.B. and Vinson, S.B. 1979a. Penetration into caterpillar cells of virus-like particles injected during oviposition by parasitoid ichneumonid wasps. Can. J. Microbiol. 25: 207-216.

Stoltz, D.B. and Vinson, S.B. 1979b. Viruses and parasitism in insects. Adv. Virus Res. 24: 125-171.

Theilmann, D.A. and Summers, M.D. 1986. Molecular analysis of *Campoletis sonorensis* virus DNA in the lepidopteran host *Heliothis virescens*. J. gen. Virol. 67: 1961-1969.

Theilmann, D.A. and Summers, M.D. 1987. Physical analysis of the *Campoletis sonorensis* virus multipartite genome and identification of a family of tandemly repeated elements. J. Virol. 61: 2589-2598.

Theilmann, D.A. and Summers, M.D. 1988. Identification and Comparison of *Campoletis sonorensis* virus transcripts expressed from four genomic segments in the insect hosts *Campoletis sonorensis* and *Heliothis virescens*. Virol. 167: 329-341.

Vinson, S.B. 1977. *Microplitis croceipes*: inhibitors of the *Heliothis zea* defense reaction to *Cardiochiles nigriceps*. Exp. Parasitol. 41: 112-117.

IDENTIFICATION AND CHARACTERIZATION OF A BACULOVIRUS ECDYSTEROID UDP-GLUCOSYL TRANSFERASE GENE

David R. O'Reilly

Departments of Entomology and Genetics
University of Georgia
Athens, GA 30602

INTRODUCTION

Control of ecdysteroid levels is of paramount importance to the regulation of insect development. In addition to control at the levels of biosynthesis and secretion, regulation of ecdysteroid metabolism is also critical for the maintenance of appropriate concentrations of these hormones. The metabolism of ecdysteroids is highly complex, and many different classes of metabolic transformation are known to occur (Koolman and Karlson, 1984). The relative importance of such reactions to the inactivation of ecdysteroids is being elucidated only now. The identification of the genes encoding enzymes involved in ecdysteroid metabolism will greatly facilitate this task. In this manuscript, the characterization of a gene (*egt*) encoding a glucosyl transferase specific for ecdysteroids is described.

IDENTIFICATION OF AcMNPV *EGT*

The *egt* gene was first discovered in a hypervariable region of the DNA genome of an insect baculovirus *Autographa californica* nuclear polyhedrosis virus (AcMNPV) (O'Reilly and Miller, 1989). Sequence analysis of this region of the AcMNPV genome revealed the presence of a 506 codon potential open reading frame of unknown function. This gene was disrupted in several of the viral mutants examined, indicating it is nonessential for viral replication (O'Reilly and Miller, 1990). Transcriptional analysis showed that the *egt* gene was transcribed early after infection of fall armyworm *Spodoptera frugiperda* IPLB SF21 cells. Two *egt*-specific transcripts, of 1.8 and 3 kb, were identified. These transcripts initiate at the same site 43 nt upstream from the *egt* translational start codon. Figure 1 summarizes the sequence and transcriptional analysis of this region.

The first indication of the function of *egt* was provided by the identification of sequences homologous to the predicted *egt* amino acid sequence in the Genbank database. *Egt* was found to be similar to several mammalian UDP-glucuronosyl transferases (Fig. 2) (O'Reilly and Miller, 1989). These enzymes catalyze the transfer of glucuronic acid to a wide variety of both exogenous and endogenous lipophilic substrates (Dutton, 1980). This conjugation reaction is of critical importance to mammals in the detoxification and safe elimination of numerous drugs and carcinogens. In addition, the normal metabolism of various endogenous compounds, such as bilirubin and steroid hormones, proceeds via their conjugation with glucuronic acid. The sequence homology suggested to us that the *egt* gene product might catalyze the conjugation and elimination of some compound in infected insects.

An interesting feature of the sequence alignments in Fig. 2 is that *egt* is 27 to 28 amino acids shorter at the C-terminal end than the glucuronosyl transferases. These enzymes are

Molecular Insect Science, Edited by
H. H. Hagedorn *et al.*, Plenum Press, New York, 1990

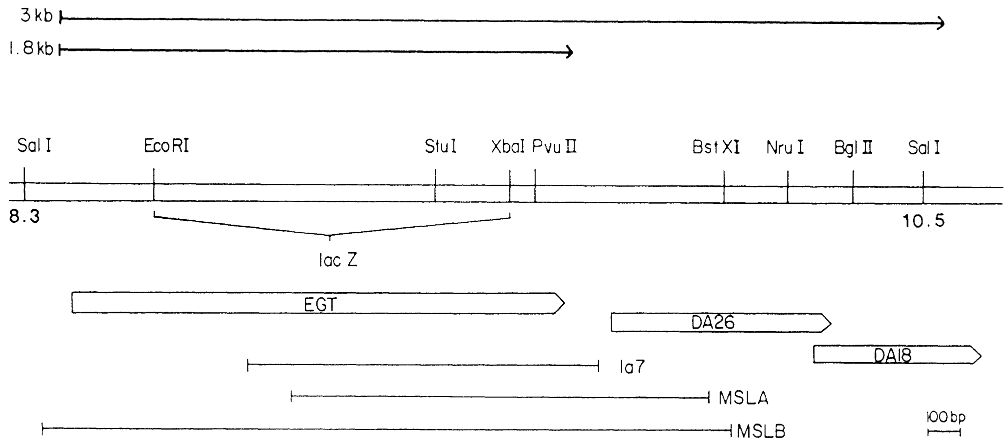


Fig. 1. Summary of sequence and transcriptional analysis of the AcMNPV *egt* gene. Open reading frames are represented by the open arrows, while the solid arrows denote the major *egt*-specific transcripts. The sequences deleted from the mutant viruses v1a7, vMSLA and vMSLB (O'Reilly and Miller, 1990; Kumar and Miller, 1987) are indicated. Sequences from the *Eco*RI site to the *Xba*I site were replaced by *lacZ* in vEGTZ, or deleted from vEGTDEL. Coordinates are given in map units.

```
E   MTILCWLALLS-----TLTAVNAANILAVFPTPAYSHHIVYKVYIEALAEKCHNVTVVKP-KLFAYS
H   *..    ***       ... . ...*-* ** *.** .  *  .. * .. *.***. .   ...
M   *      ***       . .*. ...*-* * - .** .  *. .. * .. *.***  *   .
R   *      **.       . . . ...*-* * - .** .  *. .. * .. *.***..*   *

E   TKTYCGNITEI-NADMSVE-----QYKKLVANSAMFRKRGVVSDTDTVTAANYLGLIEMFKDQF-DN
H    ..      *.    ..     -   *.**   *-   ..      .        . ....    *
M    *   *   *   . .* .       * .   . -   *.       .       . . *     *
R    *   .   ** .. .* .       * *   . -   *.       .       .   .     *

E   INVRNLIANNQ--TFDLVVVEAFADYALVFGHLYDPAPVI--QIAPGYGLAENFDTVGAVAR-HPVH
H   .. ..*. . *   **.*. .*.  .. ....*   . *   ...***..  . . .   .   **
M   .. ..*... *   **... ...*  . ....* . . .   ...*** .  . .     .   **
R   .. ..*... *   **..  ...*  . ....* . . .    ..**  *  ...     .   **

E   HPNIW-RSNFDDTEANVMTEMRLYKEF--KIL-ANMSNALLKQQFGPNTPTIEKLRNKVQLLLLNLH
H    ...  . * .   *..- .-** **  .*.  .  . .  . .*  *-*.     * .. *.
M    . .  . * .   *..- *-** .*  ...  .  ...  . .*  *-*.     . .. *.  .
R    . .  . * *   *..- *-** .*    *  .  ...  . .*  *-*..   .**.. *.

E   PIFDNNRPVPPSVQYLGGGIHLVKSAPLTKLSPVINAQMNKSKSGTIYVSFGSSIDTKSFANEFLYM
H     *.  .*. *.*...-**.*   . **  *    .- * .  ..  ..-*.** .- -. ..*   .
M     ..  .*  *.*.*.-**.*   . **  *    .- * .   .  ..-*.** .- -. ..*
R     .   .*  *.*.*.-**.*   . **  *    .- * .   .  .. *.** .- -. ..*

E   LINTFKTLDNYTILWKIDDEVVKNITLPANVITQNWFNQRAVLRHKKMAAFITQGGLQSSDEALEAG
H   . ...  . - .**..*..    . * . .-- .*. *  .* * *  ****.** ..   *. .
M   . ..   . - .***.*.     .   . *-- .*. *  .* * *  **.*.** ..  **.  *
R   . ..   . - .***.*.     .   . *-- .*. *  .* * *  **.*.** ..  **.  *

E   IPMVCLPMMGDQFYHAHKLQQLGVARALDTVTVSSDQLLVAINDVLFNAPTYKKHMAELYALINHDK
H   **** .*. .**  .   ..  * * .**  *.** .** *.. *.-* * **  .  *-. *.**.
M   ***. .** *.*  .   .   * * **.  *.*  ..* *...*.-  * ***.   *-. *.**.
R   ***. .*. ***  .   .   * * .*.  *.*  ..* *...*.-. * ***..  *-. *.**

E   ATFPPLDKAIKFTERVIRYRHDISRQLYSLKTTAANVPYSNYYMYKSVFSIVMNHLTHF#
H   .-  ***.*- *    .- **  ...*   ---* .. --- . * *-. .    *.   + 27 aa
M   .-  ***.*- *    .- **  ...* .*---. ** --- . * *-. ..   *.   + 28 aa
R   .-  ***.*- *    *- **  ...* .*---. ***--- * * *-. ..   ** * + 28 aa
```

Fig. 2. Alignment of *egt* (E) with human (H) (Jackson et. al., 1987), mouse (M) (Kimura and Owens, 1987), and rat (R) (MacKenzie, 1987) UDP-glucuronosyl transferases. Asterisks denote identical matches; dots denote conservative replacements; and hyphens denote gaps.

known to be membrane-bound and a "membrane-anchor" sequence has been identified at their extreme C-terminus (Nillson et al., 1989), in the same region missing from *egt*. Thus, it seemed likely that *egt* might be secreted from infected cells.

AcMNPV *EGT* ENCODES AN ECDYSTEROID UDP-GLUCOSYL TRANSFERASE

In insects, sugar conjugation reactions are believed to involve the transfer of glucose, rather than glucuronic acid (Smith, 1977). As in mammals, a wide variety of both exogenous and endogenous substrates are prone to conjugation. The recent identification of an ecdysteroid-glucose conjugate (Warren et al., 1986; Thompson et al., 1987) prompted us to include several ecdysteroids among the range of compounds tested as potential substrates for *egt*-mediated conjugation. The results of an experiment illustrated in Fig. 3 show that the *egt* gene product can catalyze the glucose conjugation of ecdysone. SF21 cells were infected with wild-type (wt) AcMNPV or the recombinant virus vEGTZ. This egt^- virus differs from wt AcMNPV only in the disruption of *egt* by insertion of the *Escherichia coli lacZ* gene (Fig. 1) (O'Reilly and Miller, 1989). Twelve hours post-infection the cells and extracellular medium were collected and assayed for glucose conjugation of [^{3}H]-ecdysone as described previously (O'Reilly and Miller, 1989). The data in Fig. 3 show that glucose conjugation, resulting in the formation of an ecdysone derivative of altered polarity (EG),

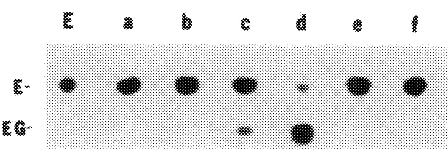


Fig. 3. AcMNPV-specific ecdysone UDP-glucosyl transferase. Cell extracts (a, c, e) or extracellular medium (b, d, f) from mock (a, b), wt AcMNPV (c, d) or vEGTZ-infected (e, f) SF21 cells were incubated in the presence of [^{3}H]-ecdysone and UDP-glucose. Reaction products were separated by thin layer chromatography. The positions of unconjugated (E) and conjugated (EG) ecdysone are shown.

occurred only when the lysate or extracellular medium of wt AcMNPV-infected cells was used (lanes c and d). The glucosyl transferase activity was found predominantly in the extracellular medium, in agreement with the sequence data (compare lanes c and d). No activity was detected in samples derived from mock-infected (lanes a and b) or vEGTZ-infected (lanes e and f) cells. The results presented in Table 1 demonstrate that, while several ecdysteroids function as substrates for this enzyme, none of the other compounds tested were conjugated to a significant extent. No conjugation was observed when UDP-glucuronic acid was used in place of UDP-glucose. These data show that the product of the *egt* gene is an ecdysteroid UDP-glucosyl transferase which is secreted into the extracellular medium by wt virus-infected cells.

EGT EXPRESSION BLOCKS MOLTING OF INFECTED INSECTS

The effects of *egt* expression on infected insects were assessed by infection of early fourth instar *S. frugiperda* or *Manduca sexta* with wt AcMNPV or egt^- mutant viruses, and monitoring for perturbations in the insects' development. The egt^- viruses used were vEGTZ (discussed above) and vEGTDEL, an AcMNPV mutant from which a large portion

of the *egt* gene has been deleted (Fig. 1). Groups of larvae were also injected with tissue culture fluid as negative controls (mock-infected larvae). The data in Table 2 show that, while all mock-infected insects molted to fifth instar, the majority of wt virus-infected larvae did not make this transition. In contrast, most larvae infected with the *egt* ⁻ viruses underwent a fourth-to-fifth instar molt. All virus-infected larvae ultimately succumbed to the infection, indicating that disruption of *egt* does not otherwise compromise AcMNPV replication, and that inhibition of molting is not merely a secondary effect of viral pathogenesis. Thus, it is clear that wt AcMNPV specifically inhibits host molting by expression of *egt*. We have obtained similar results following *per os* infection of neonate larvae, and also have observed that wt AcMNPV infection inhibits pupation (data not shown).

Table 1. Substrate specificity of *egt*

SUBSTRATE	MOCK (a)	wt (a)	vEGTZ (a)	wt (b)
p-Aminobenzoic acid	-*	-	-	-
Bilirubin	-	-	-	-
Chloramphenicol	-	-	-	-
Cholesterol	-	-	-	-
Diethylstilbestrol	-	-	-	-
Ecdysone	-	155.7	-	-
β-Estradiol	-	-	-	-
20-Hydroxyecdysone	-	87.8	-	-
Hydroxyquinoline	-	-	-	-
Makisterone A	-	89.6	-	-
- Menthol	-	-	-	-
Methylumbelliferol	-	-	-	-
α-Naphthol	-	-	-	-
p-Nitrophenol	-	-	-	-
Phenolphthalein	-	-	-	-
Testosterone	-	-	-	-
α-Tetralol	-	-	-	-

Substrates were incubated in the presence of medium from infected cells and UDP-[^{14}C]glucose (a) or UDP-[^{14}C]glucuronic acid (b). Amounts of glucose or glucuronic acid transferred (in pmol) were calculated after scintillation counting of appropriate regions of the chromatography plates. *- indicates that less than 1% of the glucose or glucuronic acid was transferred.

Table 2. Inhibition of molting by wt AcMNPV

SPECIES	VIRUS GENOTYPE	INFECTED	MOLTED
S. frugiperda	Mock	22	22
	wt	23	1
	egt ⁻	23	21
M. sexta	Mock	20	20
	wt	25	3
	egt ⁻	27	20

Early 4th instar *S. frugiperda* or *M. sexta* were injected with wt AcMNPV or *egt* ⁻ virus (vEGTZ or vEGTDEL). Mock-infected insects were injected with tissue culture fluid. Insects were monitored daily for molting behavior.

CONCLUSIONS

The data show that the baculovirus AcMNPV specifically interrupts the normal development of its insect host by producing an ecdysteroid UDP-glucosyl transferase which may be secreted into the hemolymph by infected cells. Sequence comparison of mammalian UDP-glucuronosyl transferases has shown that they belong to a family of genes which apparently evolved from a common ancestor (Jackson et al., 1987; Kimura and Owens, 1987; MacKenzie, 1987; Jackson and Burchell, 1986). These data show that AcMNPV ecdysteroid UDP-glucosyl transferase also belongs to this family. Furthermore, the AcMNPV *egt* gene product is also related to a plant UDP-glucosyl transferase (O'Reilly and Miller, 1989; Ralston et al., 1988) suggesting that UDP-glucosyl transferases in general belong to this family. It is likely, therefore, that other insect UDP-glucosyl transferases, which are responsible for the conjugation of many aglycones including various insecticides, will also be related to *egt*.

Currently, ecdysteroid-glucose conjugates have only been identified unequivocally in *M. sexta* eggs and newly hatched larvae (Warren et al., 1986; Thompson et al., 1987; Thompson et al., 1988). There is little information on the occurrence of such conjugates at other developmental stages or in other species, aside from conflicting reports derived from studies of *Calliphora vicina* (Heinrich and Hoffmeister, 1970; King, 1972). AcMNPV *egt* represents the first identified gene encoding an ecdysteroid-specific glucosyl transferase. Since baculoviruses are unique to arthropods, it is most probable that the baculovirus *egt* gene was acquired from an arthropod host during the evolution of these viruses. Thus, the AcMNPV *egt* gene is likely to be closely related to the analogous insect ecdysteroid glucosyl transferases, and its identification should facilitate the cloning of equivalent insect genes. The subsequent characterization of such insect genes will allow a thorough investigation of the role of glucose conjugation of ecdysteroids in the control of insect development.

ACKNOWLEDGMENTS

The author thanks L.K. Miller for critical reading of the manuscript; M. Brown for helpful discussions and advice on insect bioassays; and D. Perkins (USDA, Tifton, GA) for *S. frugiperda* eggs. The work described in this manuscript was supported in part by Public Health Service grant AI 23719 from the National Institute of Allergy and Infectious Disease to Lois K. Miller, and by the University of Georgia Agriculture Experiment Station.

REFERENCES

Dutton, G. 1980. "Glucuronidation of Drugs and Other Compounds." CRC Press, Boca Raton.

Heinrich, G. and Hoffmeister, H. 1970 Insect molting hormones and their mode of action. Formation of hormone glycosides as an inactivation mechanism in *Calliphora erythrocephala*. Z. Naturf. B. 25: 358-361.

Jackson, M.R. and Burchell, B.R. 1986. The full length coding sequence of rat liver androsterone UDP-glucuronyltransferase cDNA and comparison with other members of this gene family. Nucleic Acids Res. 14: 779-795.

Jackson, M.R., McCarthy, L.R., Harding, D., Wilson, S. Coughtrie, M.W.H. and Burchell, B. 1987. Cloning of a human liver microsomal UDP-glucuronosyltransferase cDNA. Biochem. J. 242: 581-588.

Kimura, T. and Owens, I.S. 1987. Mouse UDP-glucuronosyltransferase. cDNA and complete amino acid sequence and regulation. Eur. J. Biochem. 168: 515-521.

King, D.S. 1972. Metabolism of α-ecdysone and possible intermediate precursors by insects *in vivo* and *in vitro*. Gen. Comp. Endocrinol. Suppl. 3: 221-227.

Koolman, J. and Karlson, P. 1984. Regulation of ecdysteroid titer: degradation. pp 343-362 in: "Comprehensive Insect Physiology Biochemistry and Pharmacology." Vol. 7. Kerkut, G.A. and Gilbert, L.I., eds. Pergamon Press, Oxford.

Kumar, S. and Miller, L.K. 1987. Effects of serial passage of *Autographa californica* nuclear polyhedrosis virus in cell culture. Virus Res. 7: 335-349.

Mackenzie, P.I. 1987. Rat liver UDP-glucuronosyltransferase. Identification of cDNAs encoding two enzymes which glucuronidate testosterone, dihydrotestosterone, and β-estradiol. J. Biol. Chem. 262: 9744-9749.

Nillson, T, Jackson, M. and Peterson, P.A. 1989. Short cytoplasmic sequences serve as retention signals for transmembrane proteins in the endoplasmic reticulum. Cell 58: 707-718.

O'Reilly, D.R. and Miller, L.K. 1989. A baculovirus blocks insect molting by producing ecdysteroid UDP-glucosyl transferase. Science 245: 1110-1112.

O'Reilly, D.R.and Miller, L.K. 1990 Regulation of expression of a baculovirus ecdysteroid UDP-glucosyl transferase gene. J. Virol. 64: 1321-1328.

Ralston, E.J., English, J.J. and Dorner, H.K. 1988. Sequence of three bronze alleles of maize and correlation with the genetic fine structure. Genetics 119: 185-197.

Smith, J.N. 1977. Comparative detoxication of invertebrates. pp. 219-232 in: "Drug Metabolism - from Microbe to Man." Parke, D.V. and Smith, R.L., eds. Taylor and Francis, London.

Thompson, M.J., Feldlaufer, M.F., Lozano, R., Rees, H.H., Lusby, W.R., Svoboda, J.A. and Wilzer, KR. 1987. Metabolism of 26-[^{14}C]hydroxyecdysone 26-phosphate in the tobacco hornworm *Manduca sexta* L., to a new ecdysteroid conjugate: 26-[^{14}C]hydroxyecdysone 22-glucoside. Arch. Insect Biochem. Physiol. 4: 1-15.

Thompson, M.J., Svoboda, J.A., Lozano, R. and Wilzer, KR. 1988. Profile of free and conjugated ecdysteroids and ecdysteroid acids during embryonic development of *Manduca sexta* (L.) following maternal incorporation of [^{14}C]cholesterol. Arch. Insect Biochem. Physiol. 7: 157-172.

Warren, J.T., Steiner, B, Dorn, A., Pak, M. and Gilbert, L.I. 1986. Metabolism of ecdysteroids during the embryogenesis of *Manduca sexta*. J. Liq. Chromatog. 9: 1759-1782.

THE DEVELOPMENT AND RELEASE OF GENETICALLY ENGINEERED VIRAL INSECTICIDES: A PROGRESS REPORT 1986-1989

Robert D. Possee, Alison T. Merryweather, Ulrike Weyer,
Mark P.G. Harris, M. Hirst and David H.L. Bishop

Institute of Virology and Environmental Microbiology, Mansfield Road,
Oxford, OX1 3SR, United Kingdom.

INTRODUCTION

Natural virus insecticides have been used for many years to control insect pests. The baculoviruses have found particular favour for this purpose. Unlike most chemical insecticides, they affect a limited number of insect species. They have no effect on other insects, invertebrates (such as earthworms), vertebrates or plants. Furthermore, they do not pollute the environment or cause adverse reactions in soil or water. However, they are slow to kill the target insect species, often taking several days or weeks to do so. For this reason they have not gained widespread favour in modern agricultural practices. The use of chemical insecticides is preferred because they are much faster acting agents. Despite this disadvantage, in some situations baculoviruses present a viable alternative to chemicals. These include areas that are environmentally sensitive (e.g., in forests and water catchment areas), where the costs of chemicals are prohibitive or where the pest species has developed resistance to the available chemical insecticides (e.g., Cunningham, 1982; Entwistle and Evans, 1985; Podgewaite, 1985; Huber, 1986; Evans and Entwistle, 1987). Furthermore, there is now a groundswell of public opinion opposed to the use of chemicals which has added impetus to the search for alternatives. This paper discusses the use of genetic engineering to improve baculovirus insecticides and the results of a programme to introduce these agents into the environment.

Structure and replication of baculoviruses

Baculoviruses consist of rod-shaped virus particles surrounded by a protein (polyhedrin) to form crystalline structures called polyhedra. Several virus particles may be packaged in each polyhedron, as in the nuclear polyhedrosis viruses (subgroup A) or only one, as in the Granulosis viruses (subgroup B). The non-occluded baculoviruses (subgroup C) do not produce polyhedra. The target stage in the insect life cycle for the baculovirus is the larva or caterpillar. Polyhedra are ingested as the insect feeds and the polyhedrin protein surrounding the virus particle(s) is removed by the action of proteases in the alkaline environment of the caterpillar midgut. Infectious virus particles invade the midgut epithelium cells and replication of the virus begins. In Lepidoptera, for *Autographa californica* nuclear Polyhedrosis virus (AcNPV), non-occluded virus is produced in these cells and released to spread infection throughout the insect. Later in infection the virus particles are packaged within polyhedrin protein to form the virus occlusion bodies or polyhedra. Associated with the formation of polyhedra is the expression of another protein in large quantities. This is the p10 protein and is thought to play a role in the formation of an envelope around the occlusion body (Vlak *et al.*, 1988; Williams *et al.*, 1989). At this stage of the infection the insect host dies, but not before very large numbers of the polyhedra have been produced. Furthermore, prior to death the infected insects may display behav-

ioural abnormalities which are of benefit to the virus. For example, infected caterpillars may ascend to the tips of plants, thereby facilitating the distribution of polyhedra over the foliage below. Virus is released from decaying corpses and can then be spread by rainfall. Virus is also spread by animals that eat the remains of the infected insects and subsequently defaecate at other sites. Virus ingestion by birds, rodents and other beneficial insects such as carabids does not lead to infection of those species; instead the baculovirus passes directly through their intestines without inactivation.

THE POTENTIAL FOR GENETIC ENGINEERING OF BACULOVIRUSES

Genetic engineering presents the opportunity to improve the speed of action of baculovirus insecticides. It is proposed to reduce the time taken for a virus to kill the host by incorporating genes encoding insect specific toxins, insect hormones and enzymes into the virus genome. When these are expressed in the host, they are expected to have a physiological effect and possibly cause an earlier demise. The development of baculoviruses as expression vectors of prokaryotic and eukaryotic genes renders this proposal amenable to investigation. The primary example of this system, the *Autographa californica* nuclear polyhedrosis virus(AcNPV), has been recently reviewed by Luckow and Summers (1988) and Miller (1988). In cell culture, polyhedra are redundant, since only non-occluded virus is able to infect other cells. Therefore, the polyhedrin coding sequences may be deleted and replaced with those of an alternative gene without affecting virus replication (Smith *et al.*, 1983a). The foreign protein is highly expressed via the efficient functioning of the polyhedrin promoter sequences (Smith *et al.*, 1983b; Pennock *et al.*, 1984; Matsuura *et al.*, 1987). The promoter of the AcNPV p10 very late gene has also been used to express the *Escherichia coli* lacZ gene (Vlak *et al.*, 1988; Williams *et al.*, 1989).

The modification of baculovirus insecticides presents additional problems. These chiefly concern the safety of releasing genetically engineered viruses into the environment. Therefore, prior to the introduction of a virus expressing an insecticidal gene product, we have decided to assess the risks involved.

RISK ASSESSMENT OF GENETICALLY ENGINEERED VIRUSES

In the Oxford field trials with genetically engineered viruses carried out between 1986 and 1989, tests were performed at a time of the year (autumn/winter) when the natural permissive insect host was absent (infected caterpillars were supplied from the laboratory). Furthermore, the field facility used for the studies was designed to restrict the escape of infected hosts and to limit entry to the site of other insects, or rodents, moles, or larger animals that might be involved with the spread of the virus. Therefore, the risk to the environment due to the introduction of a genetically engineered virus was substantially minimized. An additional precaution was that after each field study, the site of release was disinfected and monitored to confirm that it was free from virus.

Our research programme to produce genetically engineered baculovirus insecticides is primarily concerned with the assessment of risks associated with the release of an engineered virus into a natural habitat. Therefore, we considered it extremely unwise to construct and release into the environment a genetically engineered virus that could express a foreign gene intended to alter the characteristics of that virus, without a thorough understanding of the possible consequences. An issue of particular concern was whether the host range of the engineered virus was different compared with that of the parent virus. To resolve this question extensive host range tests were undertaken before seeking permission for a field release of any modified virus. Another concern was the potential for spread of the virus by environmental conditions such as rain, or by the movement of the host species or other animals. These movements occur in natural epizootics and after application of normal viruses in the wild (Entwistle *et al.*, 1983). Therefore, the spread of engineered viruses after release was limited by constructing a netted field facility to prevent the movement of insects and other animals to and from this site.

Genetic recombination between baculoviruses

A further issue to be considered is the question of the exchange of genetic information

between the engineered virus and other species. The exchange of DNA among bacteria (involving plasmids, conjugation, etc.), is well documented. Gene exchange between genetically compatible baculoviruses may occur when two closely related baculoviruses co-infect the same host. Marker rescue studies indicate that such rates could be greater than 1% for closely related viruses (unpublished data). The likelihood of gene exchange occurring between such viruses will depend on whether the target host is already infected with a related virus, or whether another virus co-infects the same target host at the same time. The transfer of foreign DNA between baculoviruses can be analysed by studying the progeny virus recovered from dually infected cells or by appropriately designed marker rescue studies. However, so far in our studies we have not been able to identify a U.K. baculovirus that is closely related to AcNPV, or that is genetically compatible with this virus.

Genetic stability of modified viruses

Another risk factor associated with an engineered organism is the question of its genetic stability. This can be investigated by genome analysis of virus passaged in insects within the laboratory. No genetic instability has yet been identified with any of the virus constructions we have made or field tested.

Laboratory testing of genetically egineered vruses

Each genetically engineered baculovirus that has been used in field release studies was subjected to a series of laboratory analyses to resolve the issues raised above. The laboratory investigations involved the following: the construction of the candidate virus insecticide, verification of the precise genetic change by DNA sequence analysis, laboratory studies of the phenotype and genetic stability (mutability) of the viruses, host range and genetic compatibility determinations, and analyses of the physical stability of the virus in soil and on plant surfaces. Other than the expected genetic alteration designed to be incorporated into the virus genome, no unexpected change in the genome was detected and no genetic instability. The viral phenotypes (e.g. host ranges, physical stabilities) have also been as expected.

Permission for environmental releases

In each study the laboratory data were submitted to all the relevant UK regulatory bodies for independent evaluation. Particular attention was paid to the characteristics of the site proposed for the release, the insect fauna of the area, the likelihood of spread beyond the immediate ecosystem and the proposed physical constraints imposed at the release site. Permission to release the genetically engineered viruses described below were given in the form of licenses to conduct the studies under certain prescribed conditions (site arrangements, procedures to be followed for the introduction of the engineered virus, disinfection of the site after use and so on). Since baculoviruses are insecticides, the licenses were issued by the U.K. Ministry of Agriculture, Fisheries and Food (MAFF) under the Food and Environment Protection Act 1985 and Regulation of the control of Pesticides Regulations 1986. Evaluation of the proposals was made by the Health and Safety Executive Advisory Committee on Genetic Manipulation, in consultation with MAFF, the Department of the Environment and the Nature Conservancy Council. In addition, other interested parties were informed, including the Natural Environment Research Council's senior management, the owners of the field site (Oxford University), senior University authorities, the University Safety Officer, the Oxford Health and Safety Executive factory inspector, the Vale of the White Horse District Council and agencies such as "Friends of the Earth." Press coverage was also sought in national and local papers, a "Public Notice" placed in local papers. Radio and television interviews aided the process of informing the general public of the proposed studies. Therefore, a working code of practice has been established to allow the step by step risk assessment of genetically engineered organisms.

FIELD STUDIES WITH A GENETICALLY MARKED AcNPV

In field trials with natural virus insecticides it is never possible to distinguish between the applied virus and the same virus which may already be present at the site of release.

Therefore, the first release of a genetically engineered baculovirus made use of an example that had been "marked" or "tagged" with a small piece of synthetic DNA. The virus was constructed so that it could be distinguished from all other baculoviruses and genomic sequences in other organisms. The marker consisted of an oligonucleotide, 80 base pairs in length, which contained translation stop codons in all six reading frames, no ATG codon and no other genetic signals likely to affect the replication or gene product expression of the virus.

The marker element was introduced into the AcNPV genome at a position within the 3' non-coding region of the polyhedrin gene (Bishop *et al.*, 1988). Due to its placement it did not add to, or detract from, the constitution or synthesis of any viral gene products. This was confirmed by analyses of the viral phenotype (infectivity, proteins synthesized) by comparison to that of the unmodified virus.

The host species used for field releases

In the field studies it was decided to use caterpillars of the small mottled willow moth (*Spodoptera exigua*) as the target pest. This is a noctuid moth with practically a world-wide distribution; it is a seasonal immigrant to the U.K. Larvae of the moth pass through five instars. It eats a range of herbaceous dicotyledons, including sugar beet. The site chosen and used for release of the marked virus was situated in a field of light loam soil bounded by agricultural land at the Oxford University field station at Wytham, Oxfordshire. Moth species native to the region were collected at night using two ultraviolet light traps. The moths were trapped throughout the late spring, summer and autumn of 1986 and 1987.

Field release of marked AcNPV

The first field trials with the marked AcNPV were undertaken in September of 1986 (Bishop, 1986), after permission was obtained from the various bodies described above (Bishop *et al.*, 1988). Early third instar *S. exigua* larvae were fed an artificial diet containing 10 LD_{50} polyhedra equivalents of the marked AcNPV. The infected larvae (*ca* 200) were then fed a repeat dose of virus in a similar fashion. The larvae were transferred to the field site in sealed containers and placed on the sugar beet plants in the central, plexiglass enclosed area of the containment facility. Uninfected larvae were released onto nearby plants in an adjacent totally netted enclosure. One week after the release none of the infected caterpillars were alive. About 55 dead caterpillars were found on the plant surfaces. Other dead caterpillars were observed in or on the surface of the soil. Samples of soil and foliage (cabbages, sugar beet and chickweeds that grew within the facility) were taken and returned to the laboratory at 1-2 week intervals over a six month period until February 1987. These were analysed for the presence of virus.

Virus was assayed on the plant material by feeding it to permissive *T. ni* larvae (second instar) and then rearing the insects on artificial diet until pupation or death. These individuals were then monitored for the presence of polyhedra and virus DNA. Virus was identified at every sampling throughout the six month period, although not every insect fed plant material died. Virus was detected on plants that were present in the site at the initiation of the experiment (cabbages, sugar beet), as well as those that grew up in the facility after the caterpillars had died. Chickweed, recovered from outside the facility or from plants collected outside the enclosed are, did not yield virus.

The soil samples from the field site were assayed for virus by planting cabbage seeds. The derived seedlings were then fed to *T. ni* larvae and the insects reared until pupation or death, as before. The presence of the marked AcNPV was confirmed as described above. Approximately 12% of the caterpillars died of virus infections. Virus was identified at every sampling time throughout the six month period.

In the completely netted enclosure where uninfected caterpillars were placed, the larvae consumed substantial quantities of foliage for three weeks after the release and then pupated. Caterpillars were removed at the end of this period, all the plants were destroyed by autoclaving and the area was flooded with water to retrieve pupae. Virus was never isolated from these insects.

Disinfection of the field site

Six months after the field release the virus was still present in field samples confirming

that the field site remained contaminated with virus. Therefore, in February 1987, at the completion of the field experiment, the site was cleared and all foliage removed and sterilized to destroy residual virus. The site was then treated on three separate occasions with 5% formalin (twice in February and one in March). Soil samples were taken before and after each treatment and tested for virus as described above. All insects, infected or healthy, were processed to detect the presence of the marked virus. The results demonstrated that the infectious virus was present in the soil before the first formalin treatment but not after the third such treatment. These tests demonstrated that virus had survived in the soil throughout the six month period and that the site had been successfully disinfected by the formalin treatment.

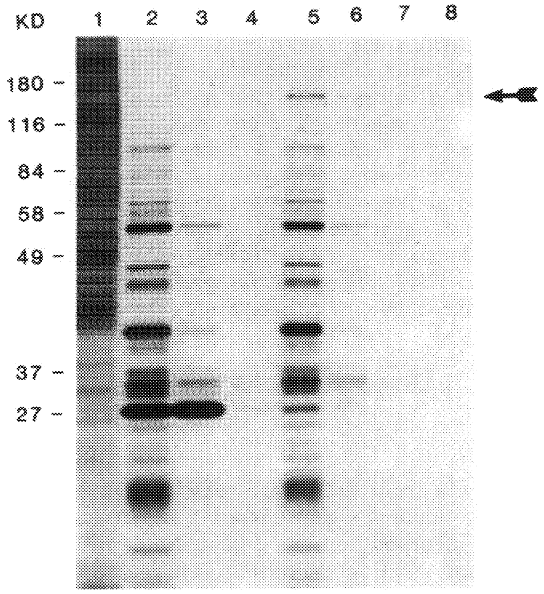


Figure 1. Expression of endotoxin by a polyhedrin-negative recombinant. *Spodoptera frugiperda* cells were infected with AcNPV (Lanes 2-4) or Ac(PH$^-$)Bt (Lanes 5-8) and pulse labelled for 1 hour at 17 (lanes 2 and 5), 24 (lanes 3 and 6), 36 (lanes 4 and 7) or 48 (lane 8) hours post-infection with [^{14}C] leucine. Cell extracts were analysed in a 12% polyacrylamide gel and detected via autoradiography. The position of the endotoxin is indicated by the arrow.

STUDIES WITH MARKED, GENETICALLY CRIPPLED AcNPV

The second release of a genetically engineered baculovirus insecticide was conducted in 1987. In this experiment, the AcNPV polyhedrin gene and its regulatory signals were removed from the virus and replaced by different synthetic oligonucleotide marker (Bishop *et al.*, 1988). The marker was positioned so that it would not affect normal virus gene expression; its sequence and position were verified by sequencing a portion of the virus genome. This time no attempt was made to exclude ATG codons from the sequence. The size and constitution of the oligonucleotide were such that it would serve only as a unique marker for the recombinant virus. No alteration to the phenotype of the virus was detected by protein analyses other than the predicted loss of the polyhedrin protein and the facility to produce polyhedra. The host range, genetic and physical stability of the recombinant virus was determined as before. The data indicate that the virus was more restricted in host range than the parent virus and that although genetically stable it was physically unstable; virus degraded rapidly in soil or on foliage.

A licence for a field release of this virus was issued by MAFF after appropriate review of the laboratory data. This release was planned and executed in the same way as the first release with a marked polyhedrin positive virus described above. The objective of this study was to determine if the polyhedrin-negative virus released from dead caterpillars persisted in the environment in soil and on plant surfaces. Caterpillars were infected in the laboratory and taken to the field site and released in the central enclosed area onto sugar beet. After one week all the caterpillars had died. Virus capable of infecting second instar *T. ni* larvae could not be identified either on the plant surfaces, or in soil samples. Even the corpses of dead caterpillars did not yield infectious virus. Therefore, these data show that the marked, so-called "crippled" or "self destruct" virus did not persist in the environment.

FIELD TRIALS USING A "FAIL SAFE" AcNPV CONTAINING A "JUNK" GENE

The polyhedrin-negative virus was considered a suitable vehicle for the expression of a foreign gene in the environment since it has a built-in safety factor. Such viruses expressing a foreign gene would degrade after killing the target insect. To investigate the expression of a foreign gene by a "crippled" virus the β-galactosidase gene from *Escherichia coli* was inserted in lieu of the polyhedrin gene. The enzyme served as a "junk" gene product and was anticipated not to affect the insects. Laboratory analyses confirmed this and demonstrated that the host range of the virus, its physical stability etc., was the same as that of the crippled AcNPV described above. The virus was also genetically stable; passage in cell culture and in insects did not cause the foreign gene to be lost. Following the granting of permission to perform field tests, infected caterpillars were placed on sugar beet plants in the field enclosure at Wytham. The subsequent course of infection was monitored confirm-ing that caterpillars died from the crippled virus containing the β-galactosidase gene.

DEVELOPMENT OF A "FAIL SAFE" AcNPV CONTAINING AN INSECT SPECIFIC TOXIN

The logical progression from the studies described above is the construction of a genetically crippled virus containing a gene encoding and insecticidal protein.

A candidate gene for incorporation into a baculovirus insecticide is the insect-specific δ-endotoxin from the gram positive bacterium *Bacillus thuringiensis*. The bacterium produces crystalline inclusions containing the toxin during sporulation. There are many *B. thuringiensis* stains with activity against a wide range of insect species (Burges, 1981). The toxin is usually active against lepidopteran larvae, but some strains affect dipteran (Goldberg and Margalit, 1977; Hall *et al.*, 1977), or coleopteran larvae (Krieg *et al.*, 1983). Natural isolates of *B. thuringiensis* have been used as insecticides (Cunningham, 1988, Luthy *et al.*, 1982). The toxin is thought to generate pores in the cell membrane leading to disruption of the osmotic balance and cell lysis (Knowles and Ellar, 1987; reviewed by Hofte and Whiteley, 1989). Specific binding sites for some *B. thuringiensis* toxins have been identified on the midgut epithelium of susceptible insects (Hoffman, *et al.*, 1988a;b). The presence of the active toxin within the gut of the insect appears to reduce feeding immediately (Heimpel and Angus, 1959). The protein is encoded either on large plasmids or in the bacterial chromosomal DNA (Gonzalez *et al.*, 1981; Schnepf and Whitely, 1981; Kronstad *et al.*, 1983; Held *et al.*, 1982; Adang *et al.*, 1985). For *B. thuringiensis* subsp. *kurstaki* HD-73 the gene is contained within a 75 kbp plasmid (Gonzalez *et al.*, 1982; Whitely *et al.*, 1982). It encodes a 130 kDa protoxin which is proteolytically cleaved to an active toxin of 68 kDa in the insect gut (Adang *et al.*, 1985). It is proposed that expression of this gene by a baculovirus within the insect might combine the speed of action of the endotoxin with the virulence of the virus and thus derive an improved virus insecticide. If the modified virus itself did not exert a direct effect, at the very least the presence of the toxin within the virus preparation might discourage the insect from feeding and reduce damage to the crop.

We have inserted the protoxin gene from *B. thuringiensis* subsp. *kurstaki* HD-73 into the genome of AcNPV to derive a polyhedrin-negative virus (Ac(PH$^-$)Bt). This we perceive to be the ideal vehicle for the cautious introduction of genetically engineered viruses into the

environment. The details of the construction of these viruses are provided elsewhere (Merryweather *et al.*, 1990).

S. frugiperda cells were infected with Ac(PH⁻)Bt, or AcNPV and pulse labelled at various times after infection. Cell extracts were analysed in 12% polyacrylamide gels and the proteins detected using autoradiography (Fig. 1). In the cells infected with Ac(PH⁻)Bt a polypeptide of about 130 kDa was detected at 17 h post infection (p.i.); at 24 h p.i. the synthesis had decreased and by 36 h p.i. it was very poorly labelled. In contrast, AcNPV produced polyhedrin protein maximally at 24 h p.i. and 36 h p.i. The 130 kDa polypeptide expressed in infected cells was identified by performing Western blot analysis with anti-endotoxin sera prepared against an intact 130 kDa endotoxin. The antiserum detected a large protein of 130 kDa, a faint component of 62 kDa, and a strong band of 44 kDa.

Table 1. Effect of expression of δ-endotoxin on feeding of *T. ni* caterpillars

		FATE OF INSECTS THAT CONSUMED DIET		
SAMPLE	REFUSED DIET	VIRAL DEATHS	NON-VIRAL DEATHS	PUPATED
No virus	0	0	0	48
AcRP8	0	25	0	24
Ac(PH⁻)Bt	50	0	0	0

Groups of approximately 50 caterpillars were fed diet containing the extract of 5000 Ac(PH⁻)Bt-or AcRP8-infected *Spodoptera frugiperda* cells and their feeding and subsequent fate monitored.

The *in vivo* activity of the δ-endotoxin was assessed by feeding extracts from Ac(PH⁻) Bt-infected cells to second instar *T. ni* larvae. Cells infected with a polyhedrin-negative recombinant lacking any foreign DNA sequences (AcRP8) were used as a control. The usual protocol in bioassays is to feed the samples to insects on a small plug of semi-synthetic diet over an 18 h period to standardize the dose. Those insects which consume the diet are then transferred to fresh, uncomtaminated diet. In tests with the Ac(PH⁻)Bt-infected cell extracts it was observed that the insects refused to consume the contaminated diet, even after extended periods of up to four days (Table 1). Similar results were obtained with intact and sonicated cells. Those insects that refused to feed were not transferred to fresh diet and died of undetermined causes, probably as a result of ingesting the endotoxin. The insects fed the AcRP8-infected cells treated in the same way consumed the dose within 18 h and were then transferred to fresh diet. Approximately 50% of the individuals fed intact or sonicated AcRP8-infected cells died, exhibiting typical symptoms of virus infection. The remainder pupated normally.

In other experiments, infected cells were treated with detergent to inactivate the virus while leaving the endotoxin unaffected. Plaque assays of these detergent-treated cells demonstrated at least a 99.5% reduction in virus titres and all insects fed the AcRP8-infected cells treated with detergent pupated normally. However, insects fed Ac(PH⁻)Bt-infected cells treated with detergent would not consume the diet, indicating the presence of biologically active endotoxin. The biological activity of the Ac(PH⁻)Bt virus alone was assessed by feeding infectious virus particles from infected cell culture medium to insects. However, a marked feeding inhibition was again observed indicating that small quantities of endotoxin were present in the preparation. It proved impossible to conduct a reliable LD_{50} determination since insects fed higher doses of virus refused to feed.

The inhibition of the feeding of insects is a typical characteristic of the natural *B. thuringiensis* product (Heimpel and Angus, 1959).

Development of a stable AcNPV containing the endotoxin

The use of a "fail safe" virus containing the endotoxin will serve as a useful tool for conducting initial environmental releases. However, the use of genetically engineered viruses will eventually have to employ stable constructions which still produce polyhedra. Therefore, a second virus was produced with the endotoxin gene inserted at a position about

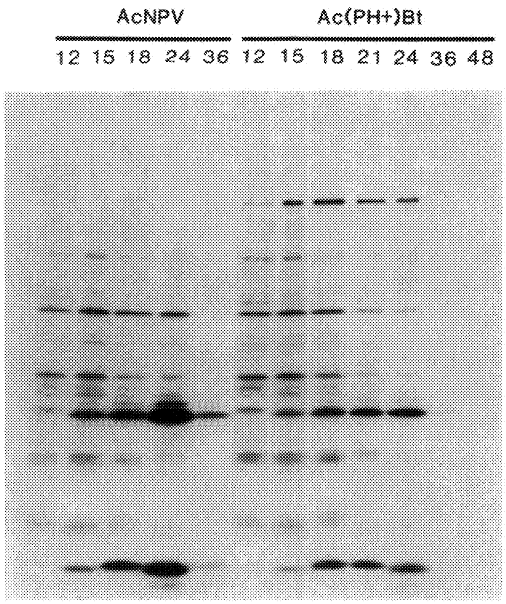


Figure 2. Expression of endotoxin by a polyhedrin positive virus. Cells were infected with AcNPV or Ac(PH$^+$)Bt and pulse labelled at 12-48 hours post infection.

92 nucleotides upstream from the polyhedrin gene. It was placed under the control of a copy of the p10 promoter (Weyer and Possee, 1988; 1989) so as to preserve the normal polyhedrin and p10 gene functions. The virus derived from these experiments (Ac(PH$^+$)Bt) produced normal polyhedra containing virus particles and grew to similar titres in cell culture.

In cells infected with Ac(PH$^+$)Bt a 130K protein was identified (Fig. 2). Interestingly the levels of polyhedrin and p10 proteins produced appeared to be lower than those synthesized by AcNPV. A similar reduction in p10 protein level was observed in cells infected with Ac(PH$^-$)Bt. This was a consistent observation in several experiments and might suggest that the intracellular endotoxin has a cytotoxic effect which consequently depresses very late gene expression. The significance of this effect has yet to be elucidated and is currently under investigation.

The biological activity of Ac(PH$^+$)Bt was assessed using bioassays. Dilutions of highly purified polyhedra derived from *T. ni* larvae infected with Ac(PH$^+$)Bt or AcNPV were fed to second instar *T. ni* to compare the LD_{50} of each virus. The LD_{50} of AcNPV was 68 polyhedra and 132 polyhedra for Ac(PH$^+$)Bt. Furthermore, the insects fed similar doses of virus died at approximately the same time. Feeding inhibition of the type seen with the Ac(PH$^+$)Bt recombinant was only observed when insects were fed very high doses of virus (>10^3 polyhedra). The LD_{50} value for Ac(PH$^+$)Bt was apparently 2-fold higher than that of the wild-type virus. We consider that this is within the experimental error of the bioassay technique used and that the two viruses probably should be regarded as having equal virulence for insect larvae. Similar variation has often been recorded when comparing the infectivity of different batches of AcNPV. While this is disappointing, considering that the aim of the study was to produce a baculovirus with increased virulence, it must also be considered that the virus is still producing biologically active δ endotoxin within the infected larvae. Therefore, after an initial round of virus replication within the infected insect, this material, released onto the plant surface as the insect decays, may have a significant effect on the larval population.

DISCUSSION

The results summarized in this report describe a programme to assess and minimize any

risk that might be involved in the release to the environment of a genetically engineered virus insecticide. It has been our intention throughout this programme to proceed in a cautious and responsible fashion, after consultation with regulatory and other interested parties. In this way we have sought to establish a procedure whereby genetically engineered baculoviruses may be brought to the point of field testing safely and with the consent of the appropriate authorities.

Several factors were considered in order to minimize risk to the environment of the proposed releases. These included the selection of the virus, the choice of the target pest, the design of the engineered viruses, the design of the field site and the procedures employed to conduct the experiment (including the final destination of the site). After the engineered viruses were constructed, they were subjected to extensive laboratory analyses. The data obtained failed to identify any measurable risk associated with the proposed experiments. This does not exclude the possibility of an unforeseen risk; however, none was identified in the laboratory studies undertaken prior to the field trials. The results obtained from the laboratory studies were used to formulate submissions to various regulatory authorities and permission was obtained to release genetically engineered viruses into the environment.

The results from the field studies confirmed the predictions made from the laboratory analyses. It was demonstrated that an innocuous genetic marker is a suitable tool for the identification of an engineered virus released into the environment. They also showed that a genetically crippled virus can be prepared that is rapidly degraded in the environment after killing its caterpillar host. This type of "fail safe" virus is a suitable candidate for further genetic engineering, and for the preparation of viruses which act more quickly than the natural virus through the incorporation of genes which determine insect specific toxins, or hormones, or other genes.

While the use of a polyhedrin-negative virus containing the endotoxin gene may not appear to be a very encouraging prospect as an improved biological control agent, it does offer other advantages. In a cautious and regulated programme to investigate the use of genetically engineered viruses in the environment it can be used in a controlled release where the long term persistence of the virus is not desired (Bishop *et al*., 1988). However, the fact that infected insects will produce active endotoxin material will permit its effect on on other insects is to be determined. Therefore, the efficacy of these agents as genetically engineered biological control agents should perhaps not be judged on the basis of preliminary laboratory studies. We are currently planning to test both viruses in enclosed systems using plants and insect larvae where their activity may be assessed in a situation more akin to the natural environment.

ACKNOWLEDGMENTS

We thank Mr. C.F. Rivers, Mr. P.H. Sterling, Mrs. M.E.K. Tinson, Mr. T.Carty, and Mr. M.L. Hirst for their assistance in collecting and/or rearing the insects for the host range studies. The genetic manipulations were undertaken in part by Dr. I. Cameron, host range tests by Ms. Cynthia Allen. In 1986 moth collections and data analyses were undertaken by Mr. S.M. Eley. In 1987 similar collections were undertaken by Ms. C. Doyle and Mr. A.C. Forkner. Carabid surveys and analyses were undertaken by Dr. J.S. Cory. Ultraviolet light measurements in the facility were conducted by Mr. H.J. Killick. The support of these and other members of the Institute's staff, particularly Mr. P.F. Entwistle, and Mr. J.S. Robertson, are gratefully acknowledged. Dr. J.I. Cooper and his staff kindly provided the plants. We are indebted to Mr. R. Broadbent and Mr. R.M. MacKenzie who constructed the field facility. We are also grateful for the advice and encouragement provided by Professor Bruce Hammock during the course of our studies with the *B. thuringiensis* δ-endotoxin and for providing the antiserum. This work was supported by NERC, the Department of the Environment and the European Community Biotechnology Action Programme.

REFERENCES

Adang, M.J., Staver, M.J., Rocheleau, T.A., Leighton, J., Barker, R.F. and Thompson, D.V. 1985. Characterized full length and truncated plasmid clones of the crystal protein of

Bacillus thuringiensis subsp. *kurstaki* HD-73 and their toxicity to *Manduca sexta*. Gene 36: 289-300.
Bishop, D.H.L. 1986. UK release of genetically marked virus. Nature 323: 496-496.
Bishop, D.H.L., Entwistle, P.F., Cameron, I.R., Allen, C.J. and Possee, R.D. 1988. Field trials with genetically-engineered baculovirus insecticides. pp 143-179 in: "The Release of Genetically-Engineered Micro-Organisms." Sussman, M., Collins, C.H., Skinner, F.A. and Stewart-Tull, D.E., eds. Academic Press, London.
Burges, H.D. ed. 1981. Microbial control of pests and plant diseases 1970-1980. Academic Press. London.
Cunningham, J.C. 1982. Field trials with baculoviruses: Control of forest insect pests. pp. 335-386 in: "Microbial and Viral Insecticides." Kurstak, E., ed. Marcel Dekker, New York.
Cunningham, J.C. 1988. Baculoviruses: Their status compared to *Bacillus thuringiensis* as microbial insecticides. Outlook on Agriculture 17: 10-17.
Entwistle, P.F., Adams, P.H.W., Evans, H.F. and Rivers, C.F. 1983. Epizootiology of a nuclear polyhedrosis virus (Baculoviridae) in European Spruce Sawfly (*Gilpinia hercyniae*): spread of disease from small epicentres in comparison with spread of baculovirus diseases on other hosts. J. Appl. Ecol. 20: 472-487.
Entwistle, P. F. and Evans, H. F. 1985. Viral control pp. 347-412 in: "Comprehensive Insect Physiology, Biochemistry and Pharmacology." Kerkut, G. A. and Gilbert, L. I., eds. Pergamon Press, Oxford.
Evans, H.F. and Entwistle, P.F. 1987. Viral diseases pp. 257-322. in: "Epizootiology of Insect Diseases." Fuxa, J.R. and Tanada, Y., eds. John Wiley, U.S.A.
Goldberg, L.J. and Margalit, J. 1977. A bacterial spore demonstrating rapid larvicidal activity against *Anopheles sergentii, Uranotaenia uniguiculata, Culex univitattus, Aedes aegypti* and *Culex pipiens*. Mosquito News 37: 355-358.
Gonzalez, J.M., Jr., Dulmage, H.T. and Carlton, B.C. 1981. Correlation between specific plasmids and delta-endotoxin production in *Bacillus thuringiensis*. Plasmid 5: 351-365.
Gonzalez, J.M., Jr., Brown, B.J. and Carlton, B.C. 1982. Transfer of *Bacillus thuringiensis* plasmids coding for δ-endotoxin among strains of *B. thuringiensis* and *B. cereus*. Proc. Natl. Acad. Sci. USA 70: 6951-6955.
Hall, I.M., Arakawa, K.Y., Dulmage, H.T. and Correa, J.A. 1977. The pathogenicity of strains of *Bacillus thuringiensis* to larvae of *Aedes* and *Culex* mosquitoes. Mosq. News 37: 246-251.
Heimpel, A.M., and Angus, T.A. 1959. The site of action of crystalliferous bacteria in Lepidoptera larvae. J. Insect Path. 1: 152-170.
Held, G.A., Bulla, L.A., Jr., Ferrari, E., Hoch, J.A., Aronson, A.I., and Minnich, S.A. 1982. Cloning and localization of the lepidopteran protoxin gene of *Bacillus thuringiensis* subs. *kurstaki*. Proc. Natl. Acad. Sci. USA 79: 6065-6069.
Hoffman, C., Luthy, P., Hutter, R. and Pliska, V. 1988a. Binding of the delta endotoxin from *Bacillus thruingiensis* to brush-border membrane vesicles of the cabbage butterfly *Pieris brassicae*. Europ. J. Bioc. 173: 85-91.
Hoffman, C., Vanderbruggen, H., Hofte, H., Van Rie, J., Jansens, S. and Van Mellaert, H. 1988b. Specificity of *Bacillus thuringiensis* δ-endotoxins is correlated with the presence of high-affinity binding sites in the brush border membrane of target insect midguts. Proc. Natl. Acad. Sci. USA 85: 7844-7848.
Hofte, H. and Whiteley, H.R. 1989. Insecticidal proteins of *Bacillus thuringiensis*. Microbiol. Rev. 53: 242-255.
Huber, J. 1986. Use of baculoviruses in pest management programs. pp. 181-190 in: "The Biology of Baculoviruses II Practical Applications for Insect Control." Granados, R.R., and Federici, B.A., eds. CRC Press, Boca Raton.
Knowles, B.H. and Ellar, D.J. 1987. Colloid-osmotic lysis is a general feature of the mechanism of *Bacillus thuringiensis* δ-endotoxins with different insect specificities. Biochem Biophys Acta 924: 509-518.
Krieg, A., Huger, A., Langenburch, G., and Schnetter, W. 1983. *Bacillus thuringiensis* var. *tenebrionis*: A new pathotype effective against larvae of Coleoptera. J. Appl. Ent. 96: 500-508.
Kronstad, J.W., Schnepf, H.E. and Whitely, H.R. 1983. Diversity of locations for *Bacillus thuringiensis* crystal protein genes. J. Bact. 154: 419-428.

Luckow, V.A. and Summers, M.D. 1988. Trends in the development of baculovirus expression vectors. Bio Tech. 6: 47-55.
Luthy, P., Cordier, J.L. and Fischer, H.M. 1982. *Bacillus thuringiensis* as a bacterial insecticide: Basic considerations and applications. pp. 35-37 in: "Microbial and Viral Pesticides." Kurstak, E., ed. Marcel Dekker, New York.
Matsuura, Y., Possee, R.D., Overton, H.A., and Bishop, D.H.L. 1987. Baculovirus expression vectors: The requirement for high level expression of protein, including glycoproteins. J. Gen. Virol. 68: 1233-1250.
Merryweather, A.T., Weyer, U., Harris, M.P.G., Hirst, M., Booth, T. and Possee, R.D. 1990. Construction of genetically engineered baculovirus insecticides containing the *Bacillus thuringiensis* subsp. *kurstaki* HD-73 delta endotoxin. J. Gen. Virol. in press.
Miller, L.K. 1988. Baculoviruses as gene expression vectors. Ann. Rev. Microbio. 42: 177-199.
Pennock, G.D., Shoemaker, C., and Miller, L.K. 1984. Strong and regulated expression of *E. coli* β-galactosidase in insect cells using a baculovirus vector. Mol. Cell. Biol. 4: 399-406.
Podgewaite, J.D. 1985. Strategies for field use of baculoviruses. pp. 775-797 in: "Viral Insecticides for Biological Control." Maramorosch, K. and Sherman, K.E., eds. Academic Press, New York.
Schnepf, H.E. and Whiteley, H.R. 1981. Cloning and expression of the *Bacillus thuringiensis* crystal protein gene. J. Biol. Chem. 260: 584-593.
Smith, G.E., Fraser, M.J. and Summers, M.D. 1983a. Molecular engineering of the *Autographa californica* nuclear prolyhedrosis virus genome: Deletion mutations within the polyhedrin gene. J. Virol. 46: 584-593.
Smith, G.E., Summers, M.D. and Fraser, M.J. 1983b. Production of human β interferon in insect cells with a baculovirus expression vector. Mol. Cell. Biol. 3: 2156-2165.
Vlak, J.M., Klinkenberg, F.A., Zaal, K.J.M., Usmany, M., Klingeroode, E.C., Geervliet J.B.F., Roosien, J and Van Lent, J.W.M. 1988. Functional analysis studies on the p10 gene of *Autographa californica* nuclear polyhedrosis virus using a recombinant expressing a p10 β-galactosidase fusion gene. J. Gen. Virol. 69: 765-776.
Weyer, U. and Possee, R.D. 1988. Functional analysis of the p10 gene 5' leader sequence of the *Autographa californica* nuclear polyhedrosis virus. Nuc. Acids Res. 16: 3635-3653.
Weyer, U., and Possee, R.D. 1989. Analysis of the promoter of the *Autographa californica* nuclear polyhedrosis virus p10 gene. J. Gen. Virol. 70: 203-208.
Whitely, H.R., Kronstad, J.W., Schnepf, H.E. and Desrosier, J.P. 1982. Cloning the crystal protein gene of *B. thuringiensis* in *E. coli.* pp. 131-144 in: "Molecular Cloning and Gene Regulation in Bacilli." Ganeson, A.T., Chang, S., and Hoch, J.A., eds. Academic Press, New York.
Williams, G.V., Rohel, D.Z., Kuzio, J., and Faulkner, P. 1989. A cytopathological investigation of *Autograph californica* nuclear polyhedrosis virus p10 gene function using insertion/deletion mutants. J. Gen. Virol. 70: 187-202.

BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF TRANSFERRIN FROM *Manduca sexta*

Neil S. Bartfeld and John H. Law

Department of Biochemistry
University of Arizona
Tucson, Arizona 85721

INTRODUCTION

While transport of macronutrients in insects (fats, carbohydrates) has received considerable attention, the transport of micronutrients is much less well studied. In the case of those insects that undergo complete metamorphosis, feeding is often limited exclusively or nearly exclusively to the larval stages. These animals must ingest and store virtually all nutrients during the larval stages and then mobilize, transport, and utilize them during pupal and adult stages.

A study of the transport and storage of iron in the larval stages of the tobacco hornworm, *Manduca sexta*, demonstrated the presence of an iron-transport protein in the hemolymph, as well as a ferritin-like storage protein in both the hemolymph and fat body (Huebers et al., 1988). The transport protein had some similarity to vertebrate transferrins, but Huebers et al. emphasized the differences, including the binding of only one ferric ion, a spectrum resembling a heme protein rather than a transferrin, and inability of the insect protein to donate iron to rabbit reticulocytes.

We isolated a similar, probably identical, protein in hemolymph of adults of the same species, but our studies indicate that this is indeed an insect transferrin. In this paper we present sequence data, derived both from Edman degradation of the intact protein and cDNA sequence, which strongly suggest a high degree of homology between the insect protein and vertebrate transferrins.

MATERIALS AND METHODS

Transferrin preparation

Transferrin was purified from the hemolymph of adult *M. sexta* by KBr density gradient ultracentrifugation gel-permeation chromatography, affinity chromatography on Cibacron Blue agarose, and chromatography on concanavalin A-Sepharose and hydroxylapatite. The final product appeared to be homogeneous by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and native PAGE.

Antibody Production

Antibodies to *M. sexta* transferrin were generated by injection of protein in polyacrylamide excised from SDS-PAGE gels into a rabbit. Booster injections were given at two-week intervals, and the rabbit was bled six weeks after the initial injection. Immunoblots were performed following SDS-PAGE of whole hemolymph and transfer to nitrocellulose (Burnette, 1981). Immunospecificity was determined using the Vectastain

Molecular Insect Science, Edited by
H. H. Hagedorn *et al.*, Plenum Press, New York, 1990

ABC kit (Vector Laboratories, Burlingame, CA) with a peroxidase detection system. Antibody titer was determined by ELISA (Engvall and Perlman, 1972).

Electrophoresis

SDS-PAGE was performed on 4-15% gradient gels by the method of Laemmli (1970), and gels were stained with Coomassie Brilliant Blue R-250. Native PAGE was performed on 4-20% gradient gels at 4°C for 17 hours at 100 volts. Protein migration under these conditions was pore-limited.

N-Terminal Sequence

The sequence of the first 34 amino acid residues of the protein was determined by Edman degradation using a Beckman 890M protein sequencer.

Site of Synthesis

Freshly dissected fat body from early wandering-stage larvae was incubated *in vitro* with [^{35}S]-methionine (New England Nuclear, Boston, MA) with rapid shaking for three hours (Prasad et al., 1986). Proteins were immunoprecipitated from the incubation medium using antitransferrin and Pansorbin *S. aureus* cells (Calbiochem, La Jolla, CA), and the products were subjected to SDS-PAGE followed by fluorography. Total RNA was extracted by the method of Chirgwin et al.(1979), followed by oligo dT-cellulose chromatography (Aviv and Leder, 1972) in order to isolate poly A^+ RNA. *In vitro* translations were performed using [^{35}S]-methionine in a rabbit reticulocyte lysate system (Promega, Madison, WI).

cDNA Library Screening

A fifth-instar day 4 amplified larval fat body λZAP II cDNA library (Stratagene Cloning Systems, La Jolla, CA) was screened with the anti-transferrin antibody using the Express-Blot alkaline phosphatase assay kit (Bio-Rad, Richmond, CA). Putatively positive plaques were further screened until pure. The insert-containing plasmids were excised *in vivo* and subjected to restriction analysis in order to determine insert size.

Message Size

RNA samples were denatured with glyoxal and DMSO by incubation at 60°C for one hour followed by electrophoresis on a 1% agarose gel. The gel was then transferred to a Nytran membrane (Schleicher and Schuell, Keene, NH) and probed with a 1.4 kilobase restriction fragment labelled to 10^8 cpm/μg using a nick- translation kit (BRL, Gaithersburg, MD). Positive plaques were visualized by autoradiography, and message size was determined by comparison to RNA standards (BRL, Gaithersburg, MD).

Partial Sequence

The 5' end of the 2.0 kilobase clone was subcloned into the vectors pTZ18U and 19U (U.S. Biochemical, Cleveland, OH) by standard procedures and partially sequenced using the chain-terminating dideoxy method (Sanger et al., 1977).

RESULTS

Preparation of Transferrin

Fig. 1 shows an SDS gel of crude hemolymph, samples obtained after each purification step, and the purified transferrin preparation. The purified protein appears to be at least 95% homogeneous.

The N-terminal sequence of transferrin is shown in Fig. 2. When the National Biomedical Research Foundation (NBRF) sequence data bank was searched in order to find proteins homologous to the 77 kDa protein, human serum transferrin was one of those pro-

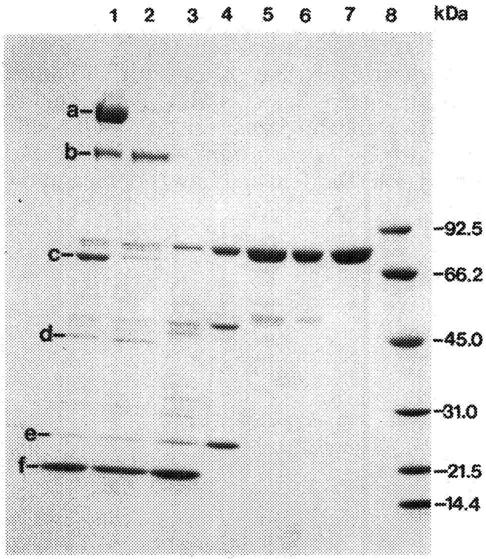


Fig. 1. Purification of transferrin from hemolymph as shown on an SDS gel. 1) Crude hemolymph. 2) After KBr density gradient ultracentrifugation. 3) After gel permeation chromatography on Biogel A 1.5. 4) After gel permeation chromatography on Sephadex G-100. 5) After Cibacron Blue dye-affinity chromatography using a 0.1-0.5 M NaCl linear gradient.. 6) After Concanavalin A-Sepharose lectin affinity chromatography using a 0-0.5 M methyl-α-D-mannopyranoside gradient.. 7) After Biogel-HT hydroxylapatite chromatography using a 0.01-0.5 M sodium phosphate gradient.*N-terminal Sequence*

```
                              10        20        30
M. sexta            AKS-------SYKLCVPAAYMKDCENMLEVPTKSKVALEKV
                    ..        .   ..  .   ..           . .
                    ...       ..  ..  . . .. ... .     ....
Human Tf            MKSVIPSDGPSVA-CVKKASYLDCIRAIAANEADAVTLDAG
                          30        40         50        60
```

Fig.2. Comparison of the N-terminal amino acid sequence of *M. sexta* transferrin with that of human transferrin. The first 34 amino acid residues were determined from a 5 nmol sample of transferrin by exhaustive Edman degradation. Sequence homology analysis was performed using the NBRF protein sequence databank. The *M. sexta* sequence is shown at the top; the human sequence at the bottom. Two dots indicate identical residues and one dot indicates a conservative substitution.

teins. The N-terminal sequence, when aligned (with the inclusion of gaps) with that of human serum transferrin whose entire sequence is known, (MacGillivray et al., 1982), contained 29% identical residues. This value rose to 56% when conservative substitutions were considered. It would be expected that the sequence at the iron-binding sites would show greater similarity owing to the presumed constraints associated with ligand binding.

Production of Antiserum

The polyclonal anti-transferrin antiserum was very specific for both larval and adult transferrins, as judged by immunoblots. No reaction was observed with preimmune serum. The antibodies were effective at greater than 10,000-fold dilution as evident by ELISA.

Site of Synthesis

The fat body was the most likely site of transferrin synthesis since the majority of hemolymph proteins are made there (Kanost et al., 1989). This was confirmed by *in vitro* fat body incubations of early wanderer larval fat body followed by immunoprecipitation (Prasad et al., 1986),. and also by *in vitro* translation of larval fat body poly A^+ RNA followed by immunoprecipitation (Bartfeld and Law, in preparation).

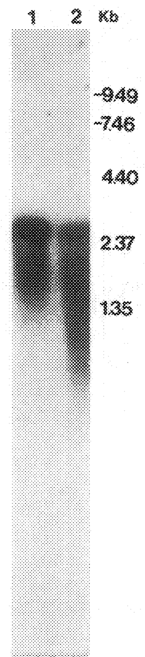


Fig. 3. Northern Blot of 10μg poly A^+ RNA (lane 1) and 20 μ g total RNA (lane 2) probed with the 1.4 kilobase cDNA fragment.

cDNA Library Screening

A fifth-instar day-4 amplified larval fat body λZAP II cDNA library was screened with the anti-transferrin antibody. Approximately 10^5 recombinants were screened, resulting in six positive plaques. Restriction analysis indicated that five of these clones were identical and 1.7 kilobases in length. The remaining clone (2.0 kilobases) hybrid-selected a message which, when translated in a rabbit reticulocyte lysate *in vivo* translation system, produced a 77 kDa protein that was immunoprecipitated with the anti-transferrin antibody (Bartfeld and Law, in preparation). A control plasmid lacking the insert did not select any message.

Message Size

A 1.4 kilobase nick-translated probe was made from the cDNA and used to probe a Northern blot containing both 10 μg of poly A^+ and 20 μg of total RNA from fifth-instar day-4 fat body. The probe hybridized to a 2.3 kilobase message in both RNA preparations (Fig. 3.) This is the same size as the message for both mouse and human transferrin (Chen and Bissell, 1987; Yang et. al., 1984).

Partial Amino Acid Sequence.

The 5' end of the 2.0 kilobase clone was subcloned and sequenced. An open reading frame was discovered and the deduced protein sequence used to search the NBRF protein database. The proteins having the highest homology to the 60 amino acid sequence segment were human transferrin (HTf), chicken transferrin (CTf), and human lactoferrin (HLf) Fig.4). The sequence was 53% homologous to human transferrin, 48% homologous to transferrin, and 35% homologous to human lactoferrin including conservative amino acid substitutions. Histidine 45 and arginine 50 are conserved among all four sequences and are thought to be residues involved in ligand binding (Crichton and Charloteaux-Wauters, 1987).

```
DFVVFQEYRT DEEPDAPFRY EAVIVVHKDL PINNLDQLKG LRSCHTGVNR NVGYKIPLTM

                450           460         470       480
CTf        KT DERP-ASY-F -AVAVARKDS NV-NWNNLKG KKSCHTAVGR TAGWVIPMGL
           ^^ ^^-^ ^-^ ^ ^^^^^^^^^- -^ ^-^^^^^ -^^^^^-^-^ -^^--^^^-^
             420          430        440        450        460
HTf           EDTPEAGY-F -AVAVVKKSA SDLTWDNLKG KKSCHTAVGR TAGWNIP
              ^^-^^^^-^ ^ ^^^^^^^^-^ -----^^^^^ -^^^^^-^-^ -^^--^^

                           230        240        250        260
LTf                      Y LAVAVVRRSD TSLTWNSVKG KKSCHTAVDR TAGWNIP
                         ^ -^^^^^^^^-- -------^^^ -^^^^^-^-^ -^^--^^
```

Fig. 4. Comparison of human transferrin, chicken transferrin and human lactoferrin with the deduced 60 amino acid segment of the 2.0 Kb cDNA. Identical or conserved residues are indicated by a ^ and nonconserved residues are indicated by a -. The conserved histidine and arginine residues are indicated by arrows. Significant areas of homology among the three proteins are boxed.

DISCUSSION

The *M. sexta* iron-binding protein resembles mammalian transferrins in many ways. The protein has a molecular weight of 77 kDas, is glycosylated, binds to Cibacron Blue, and is encoded by the same size messenger RNA. Partial sequence analysis indicates significant similarity to human transferrin, chicken transferrin, and human lactoferrin.

Huebers et. al. (1988) stated that the 80 kDa protein was probably not a transferrin, owing to its different spectral properties suggesting the presence of a heme group, its different amino acid composition, binding of one iron atom rather than two, and the lack of interaction with rat reticulocyte receptors. It is very unlikely that ferric ion is bound to a heme residue in insect transferrin, since labelled ferric ion was readily incorporated into the protein both *in vivo* and *in vitro* (Bartfeld and Law, in preparation), whereas heme proteins do not exchange their iron under physiological conditions (Hahn et al., 1940). Similarities in amino acid composition are not always indicators of homology; sequence comparisons are much more meaningful since they will reveal essential conserved residues common to all transferrins. The presence of one versus two iron-binding sites does not preclude the possibility that the second site serves another transport function. The determination of the complete amino acid sequence of the insect protein may indicate whether or not two binding sites are present.

The discovery of an insect transferrin raises several other interesting questions in addition to those bearing on protein evolution. How is iron taken up in the insect gut and transferred to hemolymph transferrin? Do insect cells use the same sort of receptor-mediated uptake of transferrin as mammalian cells (Dautry-Varsat et al., 1983), and is the apoprotein returned to the hemolymph after the iron is delivered? Insect transferrin may serve as a valuable model system for studying not only the evolution of iron-transport systems, but also as a model that may be applicable to mammalian systems. It is also possible that this system, if it proves vital to the development of functional adult flight muscle, may provide a target for interference with insect development and dispersal.

SUMMARY

An iron-binding 77 kDa glycoprotein was isolated from hemolymph of the adult sphinx moth, *Manduca sexta*. The sequence of amino acids at the N-terminus of the protein was determined through 34 residues. Comparison of this sequence with that of human serum transferrin indicated 56% similarity including conservative amino acid substitutions. An anti-transferrin antibody was used to screen a larval fat-body cDNA library from which a 2.0 kilobase clone was isolated. The labelled cDNA detected a 2.3 kilobase message on Northern blots and selected an mRNA that directed synthesis of an 77 kDa protein which was precipitated by the anti-transferrin antibody. Partial sequencing of the 5' region of the clone revealed 60 amino acids highly homologous to both human and chicken transferrins and to human lactoferrin. Based upon this and other evidence, we believe that this protein is an insect transferrin

ACKNOWLEDGMENTS

We would like to thank Mary Medina for animal raising and care. We are very grateful to Dr. Michael Kanost and Dr. Eric Hanneman for their valuable advice and assistance. This work was supported by grant GM 29238 from the National Institutes of Health.

REFERENCES

Aviv, H. and Leder, P. Purification of biologically active globin messenger RNA by chromatography on oligothymidilic acid-cellulose. Proc. Natl. Acad. Sci. USA 69: 1408-1412.

Burnette, N.W. 1981. Western blotting: electrophoretic transfer of protein from sodium dodecyl sulfate--polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. 112: 195-203.

Chen, L.-H. and Bissell, M.J. 1987. Transferrin mRNA level in the mouse mammary gland is regulated by pregnancy and extracellular matrix. J. Biol. Chem. 262: 17247-17250.

Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J. and Rutter, W.J. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18: 5294-5299.

Crichton, R.R. and Charloteaux-Wauters, M. 1987. Iron transport and storage. Eur. J. Biochem. 164: 485-506.

Dautry-Varsat, A., Ciechanover, A. and Lodish, H.F. 1983. pH and the recycling of transferrin during receptor-mediated endocytosis. Proc. Natl. Acad. Sci. USA 80: 2258-2262.

Engvall, E. and Perlman, P. 1972. Quantitation of specific antibodies by enzyme-labelled anti-immunoglobulin in antigen-coaged tubes. J. Immunol. 109: 129-135.

Hahn, P.F., Bale, W.F., Ross, J.F., Hettig, R.A. and Whipple, G.H. 1940. Radioiron in plasma does not exchange with hemoglobin iron in red cells. Science 92: 131-133.

Huebers, H.A., Huebers, E., Finch, C.A., Webb, B.A., Truman, J.W., Riddiford, L.M., Martin, A.W. and Massover, W.H. 1988. Iron binding proteins and their roles in the tobacco hornworm, *Manduca sexta* (L.). J. Comp. Physiol. B 158: 291-300.

Kanost, M.R. Kawooya, J.K., Law, J.H., Ryan, R.O., Van Heusden, M.C. and Ziegler, R. 1989. Insect haemolymph protein, in: "Advances in Insect Physiology", Academic Press, London, in press.

Laemmli, U.K. 1970. Clevage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

MacGillivray, R.T.A., Mendez, E., Sinha, S.K., Sutton, M.R., Lineback-Zins, J. and Brew, K. 1982. The complete amino acid sequence of human serum transferrin. Proc. Natl. Acad. Sci. USA 79: 2504-2508.

Prasad, S.V., Fernando-Warnakulasuriya, J.P., Sumida, M., Law, J.H. and Wells, M.A. 1986. Lipoprotein biosynthesis in the larvae of the tobacco hornworm, *Manduca sexta*. J. Biol. Chem. 261: 17174-17176.

Sanger, F., Nicklen, S. and Coulsen, A.R. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74: 5463-5467.

Yang, F., Lurn, J.B., MacGill, J.R., Moore, C.M., Naylor, S.L., Von Bragt, P.H., Baldwin, W.D., and Bowman, B.H. 19844. Human transferrin: cDNA characterization and chromosomal localization. Proc. Natl. Acad. Sci. USA 81: 2752-2756.

RECEPTOR-MEDIATED ENDOCYTOSIS OF INSECT YOLK PROTEINS

Hans-Joerg Ferenz

University of Oldenburg
Department of Biology
Oldenburg, F.R.G.

INTRODUCTION

The frontispiece of William Harvey's "De Generatione Animalium" (Concerning the Generation of Animals) published in 1651 shows the hand of Jove holding an egg, or rather an eggshell, out of which have emerged a wide variety of animals: a child, a dolphin, a spider and so on. Worked into the design is the motto "Ex ovo omnia"-- everything comes from an egg. The egg is surely one of nature's most remarkable and versatile inventions. It is a compact self-contained capsule containing everything necessary for the creation of a new life--be it a slug, a worm, a moth, a shark, an ostrich or a turtle.

The major source of nutrients for the developing embryo of all oviparous animals is the egg yolk which constitutes the non-organellar content of the oocyte. The oocyte's dramatic growth during the final stages of maturation is a result of massive deposition of yolk proteins, a process called vitellogenesis. The yolk proteins are in most animal species lipo-phospho-glyco-proteins. They are stored during oogenesis and constitute a depot of amino acids, lipid, carbohydrate and other compounds during embryogenesis. In addition considerable amounts of lipids and glycogen can be accumulated in eggs.

The process of oogenesis has been described in detail for a very large number of animals including insects. However, the molecular basis of this process, the question of "how egg yolk is made" is not well understood. As early as in 1964 Roth and Porter had already demonstrated, in electron microscopic studies on the uptake of yolk proteins by mosquito oocytes, that coated pits pinch off from the oocyte cell surface and form coated vesicles that transport extracellular fluid into the cell. Recently extensive studies on the LDL receptor system showed directly that receptor molecules clustering in coated pits is the essential event in this kind of endocytosis and thus established receptor-mediated endocytosis as a distinct mechanism for the transport of macromolecules across the plasma membrane. Subsequently many additional systems of receptor-mediated endocytosis have been defined and variations of the overall pathway have been described.

Recently, a series of studies have begun in several insect species aimed at gaining a better understanding of the cellular and molecular mechanisms involved in insect oogenesis and yolk formation. I would like to summarize this recent work with some emphasis on our studies in the locust, *Locusta migratoria*

INSECT VITELLOGENESIS

In insects the process of vitellogenesis includes the production of the yolk protein precursor(s), their transport to the ovaries and their sequestration by developing oocytes. Insect ovaries consist of ovarioles containing oocytes surrounded by a layer of follicle cells, and in some ovary types nurse cells are present. In *L. migratoria,* as well as in cockroaches and some lepidopteran species, the ovaries consist of panoistic ovarioles. In a panoistic ovariole, oocytes surrounded by a layer of follicle cells are lined up like pearls of a chain. In locusts each ovariole matures a single oocyte at a time. The penultimate oocyte

Molecular Insect Science, Edited by
H. H. Hagedorn *et al.,* Plenum Press, New York, 1990

remains at the stage of previtellogenesis until the terminal oocyte is chorionated. In locusts and cockroaches, for example, all ovarioles synchronously mature one egg each within one week. After oviposition of this batch of eggs the next oogenic cycle is initiated.

Locust eggs contain one predominant female specific yolk protein called vitellin. Its precursor in the haemolymph, vitellogenin (Vg), is synthesized as a phospho-lipo-glyco-protein in the fatbody, secreted into the haemolymph and sequestered by maturing oocytes. The Vg of *L. migratoria* has a MW of 560 kDa and contains about 8% lipids and about 13% carbohydrates. It is reassembled from two primary translation products of 225 and 235 kDa, respectively, and by treatment with SDS it can be cleaved to yield 5 subunits (Chinzei et. al., 1981). Vitellin and its precursor Vg are immunologically and electrophoretically identical. However, during uptake Vg is processed to vitellin which is less well incorporated by developing oocytes in re-uptake experiments and has an enhanced capability to aggregate (Roehrkasten and Ferenz, 1985). This is the situation we find in many insect species (Engelmann, 1979; Hagedorn and Kunkel, 1979; Kunkel and Nordin, 1985). However, in several lepidopteran species, for example, formation of protein yolk seems to be more complicated. Several proteins other than vitellogenin are deposited in substantial concentrations. Some of them are derived from the hemolymph and some from follicular epithelium (Kulakosky and Telfer, 1989). Some are not female-specific, such as lipophorin, and their role in embryonic development is unknown.

It is well-documented that in locusts Vg synthesis in the fat body is controlled by juvenile hormone (Engelmann, 1979). Juvenile hormone biosynthesis and its cyclic nature during oogenesis seem to be regulated by factors from the brain (Feyereisen, 1985) including an allatotropin in locusts, or an allatostatin in cockroaches. Additionally, factors from the ovary may also be involved in the control of juvenile hormone biosynthesis (Aden and Ferenz, unpublished data).

PATHWAY OF YOLK PROTEIN ENDOCYTOSIS

Vg is extraovarially synthesized by the fat body and then transported to the maturing oocytes and sequestered by them. To reach the oocyte surface Vg has to pass the basal membrane of the ovariole and to move through the interfollicular spaces between the follicle cells surrounding the oocytes. The yolk protein uptake is accomplished by endo-cytosis. At the oocyte surface numerous coated pits can be observed, which protrude toward the cortical ooplasma and which are pinched off as coated vesicles. Coated pits and coated vesicles consist of 3 layers: a hexagonal or pentagonal lattice meshwork on the cytoplasmatic side, the unit membrane and a coat of the yolk protein's precursor to be incorporated on the luminal side. The lattice forms a basket around the pits or vesicles of the protein clathrin. Recently, we have been able to isolate coated vesicles from maturing locust oocytes. Like coated vesicles from pig brain and chicken oocytes they have a basket structure containing clathrin as the major protein component (Roehrkasten and Ferenz, 1987b). Roth and Porter (1964) first demonstrated that Vg of *Aedes aegypti* is sequestered by developing oocytes from the maternal haemolymph by adsorptive endocytosis. Later Roth and coworkers (Roth et. al., 1976; Yusko, et. al., 1981) gave a detailed description of this process in chicken oocytes.

The uptake of insect Vg has all the characteristics of a phenomenon called receptor-mediated endocytosis, in which a membrane enclosed receptor molecule specifically binds Vg as its ligand which is then brought into the cell via the coated pits. The receptor-ligand complexes seem to concentrate in coated pits which pinch off from the oocyte membrane to form coated vesicles. The role of the clathrin lattice may be either to anchor the receptor-ligand complex to specialized membrane regions or to help to generate the force to transform a pit into a vesicle. During their movement they seem to lose their clathrin latticework and then fuse to form large yolk spheres. Some parts of the coated vesicles, clathrin lattice, membrane material and the receptor--are probably recycled, since no such protein occurs in a 1:1 ratio with Vg in the oocytes or is synthesized by the maturing oocyte. In addition, there may also exist a very large intracellular pool of receptors. At present many of these steps are not understood, but ongoing research forms a promising and important starting point to explain the process of receptor-mediated Vg endocytosis in insect oocytes.

IDENTIFICATION OF A Vg RECEPTOR

The question of whether a receptor system is involved in the specific incorporation of Vg by developing insect oocytes should be studied at the three following levels: (i) incubation of single vitellogenic follicles and measurement of the Vg uptake under various experimental conditions; (ii) isolation of oocyte membranes and study of their ability to bind specifically Vg; and, (iii) solubilization of oocyte membranes to isolate and to purify a receptor protein binding specifically Vg. So far this has been successfully done in *L. migratoria* only. However, in several insect species uptake experiments and binding studies with isolated follicle membranes clearly demonstrate the presence of Vg receptors, too.

Table 1. Uptake kinetics K_{uptake} for intact follicles of various insect species

SPECIES	K_{UPTAKE} (mM)
Locusta migratoria[1]	3.5
Hyalophora cecropia[2]	14.3
Rhodnius prolixus[3]	50.0
Aedes aegypti[4]	8.4
chicken[5]	0.1
Xenopus laevis[6]	1.5

1. Roehrkasten and Ferenz, 1985; 2. Kulakosky and Telfer, 1989; 3. Oliveira et. al., 1986; 4. A.S. Rhaikel, pers. communication; 5. Roth et. al., 1976; 6. Wallace and Jared, 1976.

Vitellogenin uptake by oocytes

Intact oocytes readily incorporate Vg under suitable *in vitro* conditions. The amount of Vg incorporated *in vitro* depends on a number of factors, among them: oocyte size, incubation temperature, pH value of the incubation medium and Vg concentration in the medium as has been shown for *L. migratoria* (Roehrkasten and Ferenz, 1985) and for several other insect species. Vg bound to the oocyte surface is rapidly incorporated. In a few cases typical saturation kinetics have been measured. In *L. migratoria* saturation was found near a Vg concentration of 45mg/ml and a K_{uptake} of 3.5 mM was calculated. Similar results were obtained for *Hyalophora cecropia* and *A. aegypti,* the data for *Rhodnius prolixus* are only indicative (Table 1). The fact that the K_{uptake} values are only in the micromolar range can be understood as an adaptation to the usually high ligand concentrations in the haemolymph. The Vg sequestration is very specific as competition experiments with bovine serum albumin and other proteins demonstrated in all insect species investigated. The endocytosis of Vg depends on metabolic energy: 2-deoxyglucose and sodium fluoride which affect glycolysis, as well as potassium cyanide and sodium azide which block cellular respiration and inhibit the endocytotic process in locust oocytes in a dose-dependent manner (Roehrkasten and Ferenz, 1985). Dinitrophenol, also an inhibitor of oxidative phosphorylation, effectively blocks endocytosis (Kulakosky and Telfer, 1987). Metabolic energy is probably needed to crosslink receptor-ligand complexes, in order to form endocytotic (coated) vesicles which move the vesicles to the yolk spheres, and unload and recycle the coated vesicle. However, little is known about this sequence of endocytosis.

Binding of vitellogenin to oocyte membranes

Oocytes surrounded by a layer of intact follicle cells are a rather complex system which does not allow a conclusive physiological characterization of a suspected Vg receptor molecule imbedded in the oocyte membrane. Thus, more direct methods have been used to detect and characterize a Vg receptor molecule in insect ovaries. Usually

membrane material from vitellogenic ovarioles is prepared and in a simple cell-free assay system the specific binding of Vg to such oocyte cell membrane suspensions is quantified. For several insect species it could be demonstrated that the ovarian membrane preparations bind Vg specifically and with high affinity (Table 2). In *L. migratoria* (Roehrkasten and Ferenz, 1986a), for example, the concentration dependence of Vg binding was determined by adding increasing amounts of labelled Vg to a constant concentration of the oocyte membrane preparation. The specific binding was then calculated by subtracting the amount of unspecifically bound Vg from the total amount of Vg bound. Unspecific or nonsaturable binding was determined by parallel incubations with large surplus of unlabeled Vg. These experiments show that the binding of Vg to locust ovarian membranes is saturable. By analyzing the results of the equilibrium saturation binding data with the Scatchard method an apparent equilibrium dissociation constant K_d of 1.092×10^{-7} M can be estimated. Similar results are reported for oocytes of the tobacco hornworm *Manduca sexta* (Osir and Law, 1986) and the cockroaches *Nauphoeta cinerea* (Koenig et. al., 1988a) and *Blattella germanica* (Koenig et. al., 1988b) (Table 2). The Vg receptors in chicken (Woods and Roth, 1984) and *Xenopus laevis* (Opresko and Wiley, 1987) oocyte membranes have a much lower affinity for Vg (Table 2). In some cases there is a close agreement between K_{uptake} and K_d. However, *a priori,* they cannot be assumed to be equal. Uptake by intact cells is a dynamic process and K_{uptake} may be influenced by many unknown rate-limiting steps. In contrast, it cannot be excluded that affinities may be altered in the preparations used for binding studies. As expected, the Vg receptors are very tissue specific: membranes of locust muscle or fat body cannot bind Vg. The receptor is a membrane protein which loses its binding activity when treated with proteolytic enzymes, such as trypsin and pronase, or when heated. The binding of Vg to its receptor and the uptake process requires calcium (Roehrkasten and Ferenz, 1986a; Osir and Law, 1986; Koenig et. al., 1988a). In the presence of the calcium chelator EGTA the binding of Vg to its receptor is reduced. Where studied, the optimum Vg binding conditions are similar to those found for uptake into oocytes. It is interesting that in *N. cinerea* two classes of Vg receptors are present in follicle-membrane preparations (Koenig et. al., 1988a). It is suggested that only the high-affinity receptor is associated with a proteoglycan secreted by the follicle cells, whereas the low affinity receptor is imbedded in the oocyte membrane.

Solubilization of the vitellogenin receptor

Based on the successful demonstration of a membrane-bound locust Vg receptor, it was possible to solubilize the Vg receptor from oocyte membranes by incubation with the non-ionic detergent octyl-b-D-glucoside. Binding studies revealed that the soluble membrane proteins contain one high-affinity compound, the locust Vg receptor, with an equilibrium dissociation constant K_d of 4.2×10^{-8}. This value and many other properties such as trypsin and heat sensitivity or pH- optimum are in agreement with previous observations at isolated locust oocyte membranes (Roehrkasten and Ferenz, 1986b). In a similar way, the Vg receptor of chicken oocytes (Stifani et. al., 1988) has recently been solubilized and characterized (Table 2). The lower affinity of Vg-binding to the chicken oocyte membrane, compared with the solubilized receptor, may be attributed to a possible contamination of the membranes with adhering yolk proteins (Stifani et. al., 1988). Interestingly, the low density lipoprotein (LDL) receptor can be isolated in a similar way and many of its biochemical properties are closely related to the Vg receptors (Schneider et. al., 1982).

The solubilized locust Vg receptor could be further purified by affinity chromatography in which Vg was covalently coupled to a gel matrix (Roehrkasten et. al., 1989). The receptor protein bound to the coupled Vg was eluted with a buffer containing suramin, a negatively charged compound, related to trypan blue, that rapidly dissociates the Vg-receptor complex (Roehrkasten and Ferenz, 1987)). Comparison of the electrophoretic pattern of ovarian membrane proteins before and after affinity chromatography showed that all the membrane proteins, except the 156 kDa protein, had been removed. The one remaining 156 kDa protein is the locust Vg receptor. The 156 kDa protein was identified as the locust Vg receptor by ligand blotting using Vg and an antibody against Vg (Fig. 1). Although a purification by a factor of 35 was achieved by affinity chromatography, some improvements concerning efficiency are necessary. Instead of affinity chromatography, somewhat better yields can be obtained by using ion exchange on DEAE-Sepharose-Cl 6B and eluting the receptor with a KCl gradient (Hafer and Ferenz, unpublished data).

Table 2. Binding activity of several insect and vertebrate membrane-bound and solubilized Vg-receptors. For comparison, data of the LDL-receptor are included.

	BINDING ACTIVITY K_d (nM)	
SPECIES	MEMBRANE-BOUND	SOLUBILIZED
Locusta migratoria	109[1]	42[2]
Manduca sexta[3]	13	n. d.
Nauphoeta cinerea[4]	296; 17[a]	n. d.
Blattella germanica[5]	79	n.d.
chicken	2.5[6]	200[7]
Xenopus laevis[8]	1.3	n. d.
LDL-receptor	40-60[9]	16-40[10]

[a]For *N. cinerea* two types of receptors with different binding activities have been reported.

1. Roehrkasten and Ferenz, 1986a; 2. Roehrkasten and Ferenz, 1986b; 3. Osir and Law, 1986; 4. Koenig et. al., 1988a; 5. Koenig et. al., 1988b; 6. Woods and Roth, 1984; 7. Stifani et. al., 1988; 8. Opresko and Wiley, 1987; 9. Schneider et. al., 1979; 10. Shneider et. al., 1982.

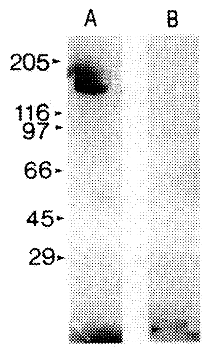


Fig. 1. Identification of the locust Vg receptor by ligand blotting. After electrophoresis the blotted receptor protein was visualized by incubation with Vg (only lane A) followed by treatment with the peroxidase-anti-peroxidase-method. The positions of molecular weight standards are indicated. (Roehrkasten et. al., 1989).

PROPERTIES OF THE LOCUST Vg RECEPTOR

The locust Vg receptor consists of one polypeptide as could be shown by electrophoretic analysis. Under non-reducing conditions and without boiling it retains its binding activity and tertiary structure resulting in a slightly reduced electrophoretical mobility. These experiments also suggest that intrachain disulphide bonds within the receptor molecule are necessary for retention of its biological activity. The receptor is a glycoprotein, but the amount and type of carbohydrates as well a their possible function are unknown. Various findings indicate that the locust Vg receptor is an extremely negatively charged protein. Isoelectric focusing of the purified Vg receptor confirmed these observations: in a gradient from pH 2.5 to pH 5.2 the receptor stops migrating at pH 3.55 (Hafer and Ferenz, unpublished data). This low isoelectric point supports the interesting observation that suramin and trypan blue, both strongly negatively charged compounds, inhibit the binding of Vg to the receptor (Roehrkasten and Ferenz, 1987a). Similar observations were made for other receptors involved in endocytosis and utilizing the coated vesicle mechanism, such as the LDL-receptor (Schneider et. al., 1982), a growth factor

receptor (Williams et. al., 1984) and the chicken Vg-receptor (Stifani et. al., 1988) (Table 3). This experimental work led to the assumption that the locust Vg-receptor apparently recognizes positive charges on the Vg molecule. Indeed already slight modification of lysine and arginine residues of the Vg molecule with various chemicals reduced the binding of Vg to its receptor (Roehrkasten and Ferenz, 1986b). These experiments suggest that some lysine residues may be essential for the specific interaction of Vg with its receptor. This has also been reported for the binding of chicken Vg (Stifani et. al., 1988) and LDL (Weisgraber et. al., 1978) to their receptors.

Table 3. Properties of the Vg receptor of *Locusta migratoria*, chicken, and the LDL-receptor

	LOCUSTA	CHICKEN[1]	LDL[2]
Molecular weight (kDa)[a]	156	96	164
Isoelectric point (pI)	3.55	n. d.	4.6
Binding activity (k_d) (solubilized form) (nM)	42	200	30
Glycoprotein	yes	n. d.	yes
Positive charges at ligand essential	yes	yes	yes

[a]Listed are the apparent molecular weights as found by SDS electrophoresis; the calculated molecular weight for the LDL receptor is about 115 kDa (Goldstein, et. al., 1985).

1. Stifani et. al., 1988; 2. Schneider, et. al., 1982.

Very recently a rabbit antibody against the locust Vg receptor was prepared. With immunocytological methods using this antibody the distribution of the Vg-receptor in maturing locust oocytes could be studied. The anti-receptor only bound to the oocyte plasma membrane; the follicular epithelium, the outer basal lamina and the dense yolk mass did not react with this antibody (Buschmann-Gebhardt and Ferenz, unpublished data). These preliminary data are in agreement with the observation of only one type of Vg-receptor in locusts located in the oocyte plasma membrane.

SUMMARY OF THE Vg RECEPTOR SYSTEM

It has clearly been established that yolk proteins are transported across the oocyte plasma membrane by a mechanism called receptor-mediated endocytosis. Studies in insects and vertebrates demonstrate that a specific surface receptor mediates the delivery of haemolymph--or blood-derived Vg into developing oocyte cells. Bound Vg rapidly enters the oocyte cell via coated pits and coated vesicles. Although in insects the understanding of the sequence of events that characterizes this endocytotic process is mainly based on electron micrographic studies, it can be assumed that it is very similar to what has been found for the LDL- and other receptor systems (Goldstein et. al., 1985). After internalization, the coated vesicles lose their clathrin coat and transient endosomes are formed. Within these endosomes the receptor dissociates from bound Vg. There is some evidence that the dissociation is triggered by a drop in pH within the endosomes: below pH 6 insect Vgs cannot be bound by their receptors; ionophores like monensin and nigericin prevent acidification of Vg endosomes mediated by ATP-driven H^+ and K^+ pumps, resulting in an inhibition of uptake and processing of Vg (Stynen et. al., 1988). Due to the acidic environment, dissociated Vg can apparently be delivered to the yolk spheres. The receptor seems to recycle back to the cell surface where it is ready to bind and internalize

new ligand molecules. However, more direct evidence for this mechanism is needed. It is assumed that the follicular epithelium surrounding each insect oocyte contributes to the selective uptake of Vg (Telfer, 1979). Recently, in the cockroach *N. cinerea*, a high-affinity Vg binding protein, presumably associated with a proteoglycan secreted by the apical surface follicle cells, was found (Koenig et. al., 1988a). This receptor seems to translocate bound Vg to a second oocyte membrane-bound receptor, which internalizes the Vg under conditions different from the haemolymph milieu. Further studies are needed to evaluate the general applicability of this observation.

At least two properties of insect Vg are essential for specific recognition by its receptor: positive charges bound to lysine residues (Roehrkasten and Ferenz, 1986b) and oligosaccharides (Koenig et. al., 1988). Their removal results in a reduction or even inhibition of Vg binding and uptake. Concerning lysine residues similar findings were reported for chicken Vg (Stifani et. al., 1988) and for apoprotein B in human LDL (Weisgraber et. al., 1978). Studies of the protein chemistry, molecular biology and cell biology of Vg receptors are just beginning. In all species studied, the receptor seems to be an integral membrane glycoprotein consisting of several domains. It will be necessary to isolate and to characterize the various domains, especially the ligand binding domain, in order to understand the function of this receptor. Vg receptors seem to be biochemically closely related to the LDL receptor. The observation that polyclonal antibodies directed against the LDL receptor cross-react to some extent with the avian Vg receptor raises the possibility that all these membrane receptors may even share some conserved structural features (Stifani et. al., 1988). Little is known about the regulation of Vg endocytosis or of the path the migrant membrane receptor and Vg have to take. Delineation of the yolk protein endocytosis and the sorting signals involved is certainly a major challenge in the field of molecular cell biology.

ACKNOWLEDGMENTS

The author wishes to thank A. Roehrkasten, J. Hafer and B. Buschmann-Gebhardt for their continuous interest in this project and their substantial contributions. Support by research grants from the Deutsche Forschungsgemeinschaft is gratefully acknowledged.

REFERENCES

Chinzei, Y., Chino, H. and Wyatt, G.R. 1981. Purification and properties of vitellogenin and vitellin from *Locustsa migratoria*. Insect Biochem. 11: 1-7.

Engelmann, F. 1979. Insect vitellogenin: Identification, biosynthesis, and role in vitellogenesis. Adv. Insect Physiol. 14: 49-108.

Feyereisen, R. 1985. Regulation of juvenile hormone titre: Synthesis. pp. 391-429 in: "Comprehensive Insect Physiology, Biochemistry and Pharmacology," Vol. 7. Kerkut, G. A. and Gilbert, L. E., eds.. Pergamon Press, New York.

Gochoco, C.H., Kunkel, J.G. and Nordin, J.H. 1988. Experimental modifications of an insect vitellin affect its structure and its uptake by oocytes. Arch. Insect Biochem. Physiol. 9: 179-199.

Goldstein, J.L., Brown, M.S., Anderson, R.G.W., Russel, D.W. and Schneider, W.J. 1985. Receptor-mediated endocytosis: Concepts emerging from the LDL-receptor system. Ann. Rev. Cell Biol. 1: 1-39.

Hagedorn, H.H. and Kunkel, J.G. 1979. Vitellin and vitellogenin in insects. Ann. Rev. Entomol. 24: 475-505.

Harvey, W. 1651. "Exercitationes de generatione animalium", Amsterdam.

Koenig, R., Kindle, H., Kunkel, J.G. and Lanzrein, B. 1988a. Vitellogenesis in the cockroach *Nauphoeta cinerea*: Separation of two classes of ovarian binding sites and calcium effects on binding and uptake. Arch. Insect Biochem. Physiol. 9: 323-337.

Koenig, R., Nordin, J.H., Gochoco, C.H. and Kunkel, J.G. 1988b. Studies on ligand recognition by vitellogenin receptors in follicle membrane preparations of the german cockroach, *Blattella germanica*. Insect Biochem. 18: 395-404.

Kulakosky, P.C. and W. H. Telfer, W.H. 1987. Selective endocytosis, *in vitro*, by ovarian follicles from *Hyalophora cecropia*. Insect Biochem. 17: 845-848.

Kulakosky, P.C. and Telfer, W.H. 1989. Kinetics of yolk precursor uptake in *Hyalophora cecropia*: Stimulation of microvitellin endocytosis by vitellogenin. Insect Biochem. 19: 367-374.

Kunkel J.G. and Nordin, J.H. 1985. Yolk proteins. pp. 83-111 in: "Comprehensive Insect Physiology, Biochemistry and Pharmacology," Vol. 8. Kerkut, G. A. and Gilbert, L. I., eds. Pergamon Press, New York.

Oliveira, P.L., Katia, C.G., Damiao, M.G. and Hatisaburo, M. 1986. Uptake of yolk proteins in *Rhodnius prolixus*. J. Insect Physiol. 32: 859-866.

Opresko, L.K. and Wiley, H.S. 1987. Receptor-mediated endocytosis in *Xenopus* oocytes. J. Biol. Chem. 262: 4109-4115.

Osir, E.O. and Law, J.H. 1986. Studies on binding and uptake of vitellogenin by follicles of the tobacco hornworm, *Manduca sexta*. Arch. Insect Biochem. Physiol. 3: 513-528.

Roehrkasten, A. and Ferenz, H.-J. 1985. *In vitro* study of the selective endocytosis of vitellogenin by locust oocytes. Wilhelm Roux's Arch 195: 411-416.

Roehrkasten, A. and Ferenz, H.-J. 1986a. Properties of the vitellogenin receptor of isolated locust oocyte membranes. Int. J. Inv. Reprod. Dev. 10: 133-142.

Roehrkasten, A. and Ferenz, H.-J. 1986b. Solubilization of the locust vitellogenin receptor. Biochem. Biophys. Acta 860: 577-582.

Roehrkasten, A. and Ferenz, H.-J. 1987a. Inhibition of yolk formation in locust oocytes by trypan blue and suramin. Wilhelm Roux's Arch. 196: 165-168.

Roehrkasten, A. and Ferenz, H.-J. 1987b. Coated vesicles from locust oocytes: Isolation and characterization. Int. J. Inv. Reprod. 12: 341-346.

Roehrkasten, A., Ferenz, H.-J., Buschmann-Gebhardt, B. and Hafer, J. 1989. Isolation of the vitellogenin-binding protein from locust ovaries. Arch. Insect Biochem. Physiol. 10: 141-149.

Roth, T.F., Cutting, J.A. and Atlas, S.B. 1976. Protein transport: A selective membrane mechanism. J. Supramol. Struct. 4: 527-548.

Roth, T.F. and Porter, K.R. 1964. Yolk protein uptake in the oocyte of the mosquito *Aedes aegypti*. J. Cell Biol. 20: 313-332.

Schneider, W.J., Basu, S.K., McPhaul, M.J., Goldstein, J.L. and Brown, M.S. 1979. Solubilization of the low density lipoprotein receptor. Proc. Natl. Acad. Sci. USA 76: 5577-5581.

Schneider, W.J., Beisiegel, U., Goldstein, J.L. and Brown, M.S. 1982. Purification of the low density lipoprotein receptor, an acidic glycoprotein of 164,000 molecular weight. J. Biol. Chem. 257: 2664-2673.

Stifani, S., George, R. and Schneider, W.J. 1988. Solubilization and characterization of the chicken oocyte vitellogenin receptor. Biochem. J. 250: 467-475.

Stynen, D. Woodruff, R.I. and Telfer, W.H. 1988. Effects of ionophores on vitellogenin uptake by *Hyalophora* oocytes. Arch. Insect Biochem. Physiol. 8: 261-276.

Telfer, W.H. 1979. Sulfate and glucosamine labelling of the intercellular matrix in vitellogenic follicles of a moth. Roux's Arch. Dev. Biol. 185: 347-362.

Wallace, R. and Jared, D.W. 1976. Protein incorporation by isolated amphibian oocytes. V. Specificity for vitellogenin incorporation. J. Cell Biol. 69: 345-351.

Weisgraber, K.H., Innerarity, T.L. and Mahley, R.W. 1978. Role of lysine residues of plasma lipoproteins in high affinity binding to cell surface receptors on human fibroblasts. J. Biol. Chem. 253: 9053-9062.

Williams, L.T., Tremble, P.M., Lavin, M.F. and Sunday, M. 1984. Platelet-derived growth factor receptors from high affinity state in membrane preparation. J. Biol. Chem. 259: 5287-5294.

Woods, J.W. and Roth, T.F. 1984. A specific subunit of vitellogenin that mediates receptor binding. Biochemistry 23: 5774-5779.

Yusko, S., Roth, T.F. and Smith, T. 1981. Receptor-mediated vitellogenin binding to chicken oocytes. Biochem. J. 200: 43-50.

SERINE PROTEASE INHIBITORS FROM THE SERPIN GENE FAMILY IN *Manduca sexta* AND *Drosophila melanogaster*

Michael R. Kanost

Department of Biochemistry and Center for Insect Science
University of Arizona
Tucson, AZ 85721

INTRODUCTION

The hemolymph of lepidopteran insects has been shown to contain two classes of proteins that inhibit serine proteases (reviewed in Kanost et al., 1990). Low molecular weight proteins (7-9 kDa) from both *Bombyx mori* (Sasaki, 1984) and *Manduca sexta* (Ramesh et al., 1988) have been shown to be homologous with the vertebrate Kunitz-type inhibitors such as pancreatic trypsin inhibitor. A group of larger proteins (42-47 kDa) are members of the serpin family of serine protease inhibitors (Kanost et al., 1989). The serpins include mammalian serum proteins such as α1-antitrypsin, antithrombin, α2-antiplasmin, and plasminogen activator inhibitor (Boswell and Carrell, 1988). A trypsin inhibitor (42 kDa) and a chymotrypsin inhibitor (43 kDa) from hemolymph of *B. mori* larvae (Sasaki and Kobayashi, 1984; Eguchi and Shomoto, 1985) have properties similar to the vertebrate serpins, including a mechanism in which the enzyme and inhibitor form a stable complex after cleavage of a reactive site near the inhibitor's COOH-terminal (Sasaki, 1985; Sasaki et al., 1987).

We have recently cloned a serpin cDNA from a *M. sexta* fat body cDNA library (Kanost et al., 1989). This cDNA encodes a hemolymph glycoprotein of 47 kDa which inhibits vertebrate elastase. The amino acid sequence of the protein is 20-30% identical with those of the vertebrate serpins, with highest similarity to human antithrombin and plasminogen activator inhibitor. The reactive site of the protein has an alanine residue at its P1 position, determining its specificity as an elastase inhibitor. The protein has therefore been named *Manduca* alaserpin, following a convention of naming serpins of unknown function based on the amino acid at the P1 position. *Manduca* alaserpin is present in hemolymph of day 3 fifth instar larvae at 430 µg/ml, which would result in a half-life of 0.01 seconds for elastase in hemolymph, a reaction rapid enough to indicate that alaserpin could efficiently regulate elastase-type enzymes in hemolymph (Kanost et al., 1990).

Four different serpin proteins have now been isolated from hemolymph of *M. sexta* larvae (Kanost, 1990). These include the alaserpin as well as two chymotrypsin inhibitors and a trypsin inhibitor, all of approximately the same molecular weight and with isoelectric points of 4.4 to 4.8. These four proteins appear to be the major serpins in *M. sexta* larval hemolymph, but less abundant inhibitors of similar size and charge are also present. The aims of the experiments described here were to estimate the number of serpin proteins in *M. sexta* hemolymph, to investigate some potential functions for serpins in insects, and to show that serpins are present in *Drosophila melanogaster* as a first step toward the use of genetics to study the biological roles of insect serpins.

Molecular Insect Science, Edited by
H. H. Hagedorn *et al.*, Plenum Press, New York, 1990

MATERIALS AND METHODS

Insects

M. sexta eggs were supplied by Dr. J. Buckner, United States Department of Agriculture, Fargo, ND. Larvae were reared as described by Prasad et al. (1986). *D. melanogaster* larvae were Canton S wild type strain reared on instant medium (Carolina Biological Supply).

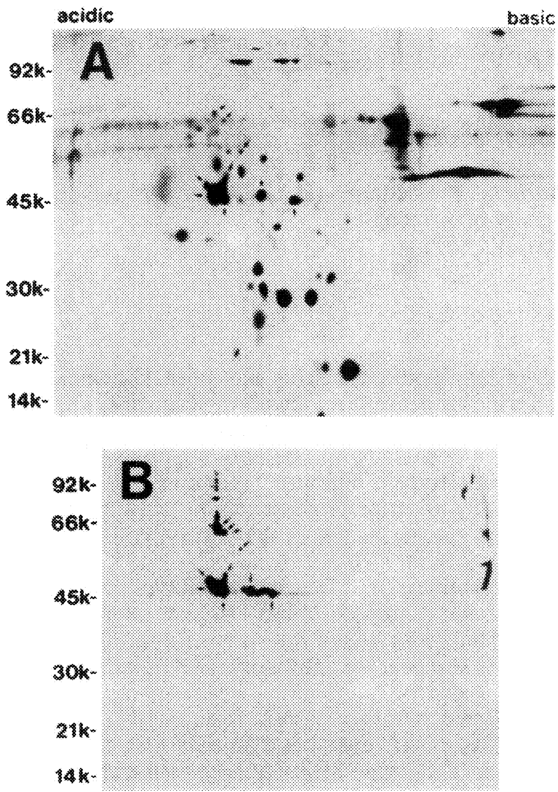


Fig. 1. Two dimensional gel electrophoresis of *Manduca sexta* hemolymph proteins. Proteins in 10 μl of hemolymph from a day 2 fifth instar larva were first separated by isoelectric focusing using pH 3.5-10 carrier ampholytes, then by SDS polyacrylamide gel electrophoresis in a 10% acrylamide gel. In (A) the gel was stained with silver. In (B) the proteins in a duplicate gel were transferred to nitrocellulose and detected with rabbit antiserum to *M. sexta* alaserpin and goat anti-rabbit IgG conjugated to alkaline phosphatase. Arrows indicate major proteins observed in both gels.

Electrophoretic Analysis of Proteins

One dimensional SDS-polyacrylamide gel electrophoresis was carried out in a 10% acrylamide gel (Laemmli, 1970) and proteins were stained with Coomassie Blue. Two dimensional gel electrophoresis was performed as described by O'Farrell (1975). In the first dimension, isoelectric focusing was carried out using pH 3.5-10 carrier ampholytes (LKB), and in the second dimension proteins were separated by SDS polyacrylamide gel electrophoresis in a 10% acrylamide gel. One gel was stained with silver (Heukeshoven and Dernick, 1985), and proteins in a duplicate gel were transferred to nitrocellulose. For immunoblots from both one and two dimensional gels, hemolymph proteins that reacted with rabbit antiserum to *M. sexta* alaserpin (Kanost et al., 1989) were visualized with goat anti-rabbit IgG conjugated to alkaline phosphatase (BioRad).

Rabbit IgG Isolation

IgG fractions from rabbit antiserum to *Manduca* alaserpin and from pre-immune serum from the same rabbit were isolated using a column of DEAE Affi-Gel Blue (Bio-Rad) following manufacturer's instructions. The resulting IgG was in a solution of 20 mM Tris-HCl, pH 8.0, 28 mM NaCl.

Phenoloxidase Assay

Hemolymph was collected from a cut proleg of chilled *M. sexta* larvae, and hemocytes were removed by centrifugation (11,000xg, 5 min, 4 °C). Phenoloxidase activity was assayed by adding 25 µl cell free hemolymph to 1 ml 5 mM dopamine in 20 mM ammonium acetate, pH 6.0 and measuring the increase in absorbance at 410 nm for 5 min.

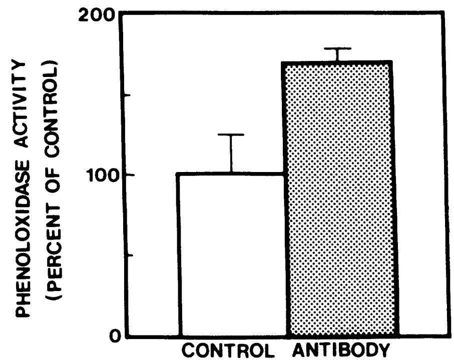


Fig. 2. Effect of alaserpin antibody on activation of phenoloxidase. Phenoloxidase activity was measured in hemolymph of *Manduca sexta* larvae (fifth instar, day one) previously injected with either IgG from alaserpin antiserum (ANTIBODY) or from preimmune antiserum (CONTROL). Error bars represent mean ± standard error (n=5).

Fat Body and Hemocyte Culture

Fat body from day 4 fifth instar *M. sexta* larvae was carefully dissected and washed three times for two minutes in 1 ml cell culture medium (Excell 400 medium (J.R. Scientific) containing 50 µg/ml gentamycin sulfate and 2.5 µg/ml amphotericin B). Hemocytes were collected from chilled day 4 fifth instar larvae and day 2 pupae by pericardial puncture (Horohov and Dunn, 1982) into a polypropylene tube. Hemocytes were pelleted by centrifugation (100xg, 10 min, 24 °C), washed once in 1 ml cell culture medium by gentle resuspension and centrifugation as above, and resuspended in 1 ml medium. Hemocyte concentrations were determined using a hemocytometer and adjusted to 10^5 cells/ml by dilution with an appropriate amount of medium. Granulocytes and plasmatocytes were the predominant hemocyte types in these preparations. Cultures were established by placing approximately 1/8 of the fat body tissue from the same animal (50 mg wet weight) in individual wells of a 24 well tissue culture dish (Falcon 3047) containing 1 ml medium per well. To the wells were then added 10^4 larval or pupal hemocytes in 100 µl medium and either 100 µg *Manduca* alaserpin or chymotrypsin inhibitor 1 (Kanost, 1990) in 100 µl buffer (50 mM Tris-HCl, pH 7.5) or 100 µl buffer alone. The cultures were incubated for 24 hr at 24°C with shaking at 130 rpm.

Protease Assay

Protease activity in culture medium was detected by a casein-agarose gel assay (Bio-Rad) using 15 µl medium per sample well and stopping the reaction after 24 hr.

Gel Filtration Chromatography of Drosophila Serum

Hemolymph was collected from third instar *D. melanogaster* larvae by puncturing the cuticle with a 30 gauge needle, while avoiding injury of the gut, and collecting hemolymph with a micropipette. Hemolymph was then transferred to an ice cold microcentrifuge tube containing a few crystals of phenylthiourea. Hemocytes were removed by centrifugation (11,000xg, 5 min, 4°C) and the serum (200 µl) was applied to a column (1 x 180 cm) of Sephacryl S-200HR (Pharmacia). The column was eluted with 50 mM Tris-HCl, pH 7.5, and 4 ml fractions were collected at 0.3 ml/min. Chymotrypsin inhibitor activity in column fractions was detected as described previously (Kanost, 1990) except that 0.25 µg instead of 1.0 µg chymotrypsin was used for each sample tested.

RESULTS

Approximately ten serpin proteins are present in M. sexta hemolymph

During purification of the four major serpins in *M. sexta* hemolymph (Kanost, 1990) several other less abundant protease inhibitors of the same size were also detected. To learn how many hemolymph proteins that might be serpins are present in *M. sexta*, serum proteins were analyzed by two dimensional gel electrophoresis and immunoblotting (Fig. 1). Silver staining revealed a cluster of approximately ten spots which represent acidic proteins of 45-48 kDa that were also detected by the antiserum to alaserpin. Several other larger proteins (55-100 kDa) were also detected by immunoblotting. Although it is possible that some of these spots may represent the same polypeptides with differences in glycosylation, the indication from this experiment is that insect hemolymph might be similar to human blood in containing a large number of serpin proteins. The higher molecular weight immunoreactive proteins may represent complexes of serpins with serine proteases (Sasaki et al, 1987).

Hemolymph serpins and regulation of prophenoloxidase activation

One potential function for the serpins in insects is regulation of the serine proteases involved in conversion of prophenoloxidase to active phenoloxidase (Leonard et al., 1985; Yoshida and Ashida, 1986; Saul and Sugumaran, 1987). Saul and Sugumaran (1986) have shown that the low molecular weight protease inhibitors in *M. sexta* hemolymph can regulate prophenoloxidase activation. To test whether the serpins may also be involved in regulation of this pathway, an experiment was performed in which antibodies to alaserpin were used in an attempt to inactivate *M. sexta* serpins *in vivo*. To separate antibodies from the endogenous serpins in rabbit serum, IgG fractions were isolated from antiserum to alaserpin and from serum from the same rabbit before immunization. Day one fifth instar larvae were injected with 150 µg of immune or pre-immune IgG. Thirty minutes after injection, hemolymph was collected from a cut proleg into a microcentrifuge tube, incubated for 5 min at room temperature, and then assayed for phenoloxidase activity. Hemolymph from larvae injected with antibodies to alaserpin had significantly higher phenoloxidase activity (t test, $\alpha<0.05$) than hemolymph from control animals. (Fig. 2). It appears that inactivation of hemolymph serpins by the antibodies may have led to a decreased capability for regulating the activation of prophenoloxidase during bleeding. Further experiments with isolated components of the prophenoloxidase activation pathway and purified serpins should help to define the regulatory role of individual hemolymph serpins in this process.

Serine proteases secreted by hemocytes in vitro

During metamorphosis of holometabolous insects a great deal of cell replacement and reorganization occurs. It seems reasonable to suggest that proteases might be required for these remodelling processes. Kurata et al. (1989) recently demonstrated that incubation of larval or early pupal fat body with pupal hemocytes of *Sarcophaga peregrina* results in dissociation of the fat body tissue, perhaps mimicking the process that occurs *in vivo* during metamorphosis. The dissociation *in vitro* required Ca^{2+}, which the authors suggested might be a cofactor for a protease. I have repeated some of the same experiments with fat body and hemocytes of *M. sexta* and also found that incubation of fat body with pupal hemocytes

results in dissociation of the tissue (Kanost, unpublished data). Protease activity is present in the medium of hemocyte + fat body cultures but not generally in cultures of either hemocytes or fat body alone. In most experiments protease activity is greater in medium from cultures containing pupal hemocytes than larval hemocytes, and in every case the protease activity can be inhibited by alaserpin or chymotrypsin inhibitor 1 from *M. sexta* hemolymph (Fig. 3). One function of the hemolymph serpins may be to regulate digestion by proteases released from hemocytes during metamorphosis.

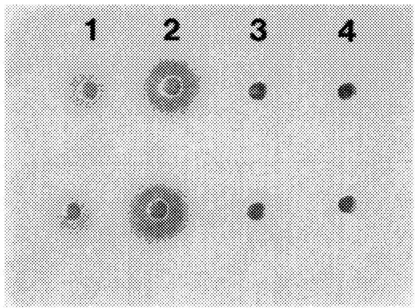


Fig. 3. Protease activity in medium from cultures of larval fat body with larval or pupal hemocytes. Protease activity in 15 µl of medium was assayed by digestion of casein in an agarose gel. The clear area around each sample well is proportional to the amount of protease in the sample. 1) medium from fat body + larval hemocytes; 2) medium from fat body + pupal hemocytes; 3) medium from fat body + pupal hemocytes + alaserpin; 4) medium from fat body + pupal hemocytes + chymotrypsin inhibitor 1.

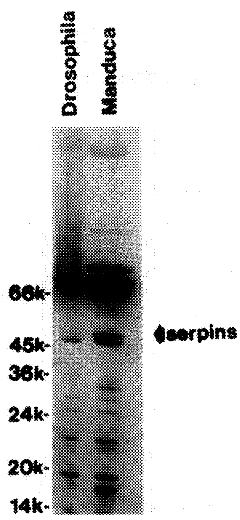


Fig. 4. Hemolymph proteins of Manduca sexta and *Drosophila melanogaster*. Samples of hemolymph (2 µl) from third instar *D. melanogaster* larvae and from fifth instar *Manduca sexta* larvae were analyzed by SDS polyacrylamide gel electrophoresis.

Serpins in Hemolymph of Drosophila

The physiological functions of many of the human serpins have been learned from individuals with a genetic deficiency in one of the serpin genes which leads to a specific disease. For example, deficient antithrombin results in bleeding problems, and insufficient regulation of neutrophil elastase by α1-antitrypsin leads to emphysema. Study of pheno-

types resulting from mutations in insect serpin genes might similarly elucidate the biological roles of these proteins. As a first step toward this objective, I have begun to investigate the serpins in hemolymph of *D. melanogaster*.

Preliminary experiments have shown that inhibitors of elastase, chymotrypsin, along with lower levels of trypsin inhibitor are present in hemolymph of *D. melanogaster* larvae. Analysis by SDS polyacrylamide gel electrophoresis showed that hemolymph of *D. melanogaster* contains at least one polypeptide that co-migrates with the *M. sexta* serpins (Fig. 4). This band reacts weakly on Western blots with antisera to the *M. sexta* serpins (Kanost, unpublished data). Analysis of hemolymph proteins by gel filtration chromatography demonstrated that two peaks of chymotrypsin inhibitor activity can be separated (Fig. 5). The first peak contains proteins with a native molecular weight of approximately 50,000, consistent with the size of serpin proteins. Confirmation that these proteins are members of the serpin family awaits further characterization, including sequence analysis.

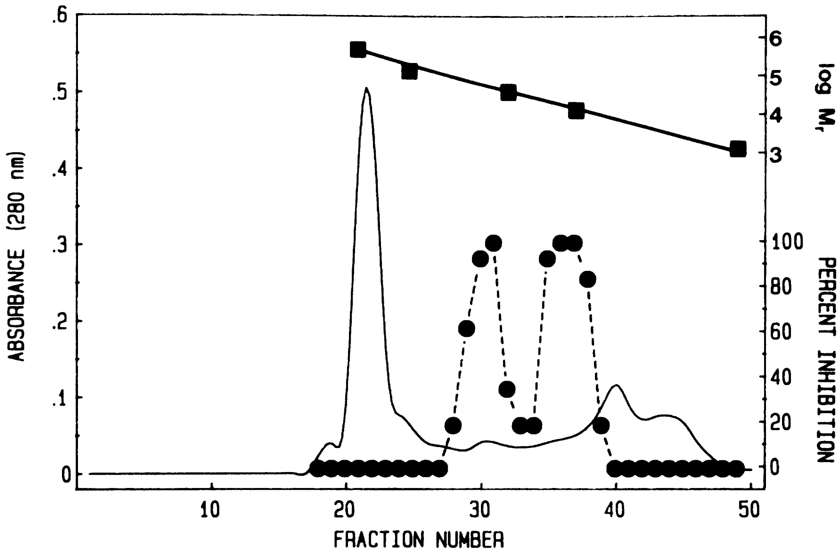


Fig. 5. Gel filtration chromatography of *Drosophila melanogaster* hemolymph proteins. A sample of cell free hemolymph (200 µl) from third instar larvae was applied to a column of Sephacryl S-200HR and eluted with 50 mM Tris-HCl, pH 7.5. Solid line: absorbance at 280 nm. Circles: percent inhibition of 0.25 µg chymotrypsin. Squares: elution volumes of standard proteins used as molecular weight markers (thyroglobulin, 670 kDa; gamma globulin, 158 kDa; ovalbumin, 44 kDa; myoglobin, 17 kDa; vitamin B-12, 1.3 kDa).

DISCUSSION

Hemolymph of *M. sexta* contains a number of proteins that are members of the serpin gene family. Four have been purified and shown to be inhibitors of serine proteases, with specificities for different types of enzymes, but their targets *in vivo* are still unknown. It may be that some of the insect serpins have functions unrelated to protease inhibition, as have human angiotensinogen, thyroxine and corticosteroid binding globulins, and chicken ovalbumin, all members of the serpin gene family. It will be interesting to learn whether serpin genes in insects have evolved to take on an array of functions as diverse as those in vertebrates.

Several kinds of biological functions can be imagined for serine protease inhibitors in insects. The prophenoloxidase activation pathway is a prime candidate for regulation by the serpins or the unrelated Kunitz-type inhibitors or by some combination of the two. There may also be other pathways in insects that use cascades of serine proteases that have not yet been recognized. The inhibitors could also help to protect insects from proteases produced by pathogens, particularly some fungi in which serine proteases are involved in penetration

of the cuticle (St. Leger et al., 1988). Protease inhibitors in hemolymph might protect tissues from damage due to accidental leakage of gut or molting fluid proteases into the hemocoel; the *M. sexta* serpins are able to inhibit midgut trypsin and chymotrypsin (Kanost, unpublished data).

Serpins could also have a several roles in developmental biology. The *snake* and *easter* genes of *D. melanogaster*, involved in dorsal-ventral pattern formation in embryos, appear by sequence homology to be serine proteases (Chasan and Anderson, 1989), and the activity of these may need to be regulated. Serine proteases and inhibitors are also involved in eversion of imaginal discs in *D. melanogaster* (Pino-Heiss and Schubiger, 1989). Serpins might regulate other types of cell movement and reorganization during metamorphosis, such as dissociation of larval tissues prior to remodelling or replacement by adult tissues. The metamorphosis of the nervous system could involve serpins which regulate neurite growth, as glia-derived nexin, a serpin, does in vertebrates (Monard, 1988). We have recently detected serpin activity, immunoreactivity, and mRNA in brains of *M. sexta* pupae (Hanneman and Kanost, unpublished data).

The functions of serpins in insect biology might best be explored with the powerful methods available in *Drosophila* genetics. The preliminary results reported here on serpins in *Drosophila* should provide a basis for further experiments involving cloning of serpin genes and eventually the isolation of mutants.

ACKNOWLEDGMENTS

I thank Martina Biczkowski-Gudarzi for help with two dimensional electrophoresis and Dr. Michael Wells for the use of laboratory facilities. This research was supported by National Institutes of Health Grants GM41247 and HL39116.

REFERENCES

Boswell, D.R. and Carrell, R.W. 1988. Genetic engineering and the serpins. Bioessays 8: 83-87.

Chasan, R. and Anderson, K. 1989. The role of *easter*, an apparent serine protease, in organizing the dorsal-ventral pattern of the Drosophila embryo. Cell 56: 391-400.

Eguchi, M. and Shomoto, K. 1985. Purification and properties of a chymotrypsin inhibitor from the silkworm haemolymph. Comp. Biochem. Physiol. 81B: 301-307.

Heukeshoven, J. and Dernick, R. 1985. Simplified method of silverstaining in polyacrylamide gels and the mechanism of silver staining. Electrophoresis 6: 103-112.

Horohov, D.W. and Dunn, P.E. 1982. Changes in the circulating hemocyte population of *Manduca sexta* larvae following injection of bacteria. J. Invertebr. Pathol. 40: 327-339.

Kanost, M.R. 1990. Isolation and characterization of four serine proteinase inhibitors (serpins) from hemolymph of *Manduca sexta*. Insect Biochem. In press.

Kanost, M.R., Kawooywa, J.K., Law, J.H., Ryan, R.O., Van Heusden M.C., and Ziegler, R. 1990. Insect Hemolymph Proteins. Adv. Insect Physiol. In press.

Kanost, M.R., Prasad, S.V., and Wells, M.R. 1989. Primary structure of a member of a member of the serpin superfamily of proteinase inhibitors from an insect, *Manduca sexta*. J. Biol. Chem. 264: 965-972.

Kurata, S., Komano, H., and Natori, S. 1989. Dissociation of *Sarcophaga peregrina* (flesh fly) fat body by pupal haemocytes *in vitro*. J. Insect Physiol. 35: 559-565.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 226: 680-685.

Leonard, C., Söderhäll, K., and Ratcliffe, N.A. 1985. Studies on prophenoloxidase and protease activity of *Blaberus craniifer* haemocytes. Insect Biochem. 15: 803-810.

Monard, D. 1988. Cell-derived proteases and protease inhibitors as regulators of neurite outgrowth. Trends Neurosci. 11: 541-544.

O'Farrell, P.H. 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. 250: 4007-4021.

Pino-Heiss, S. and Schubiger, G. 1989. Extracellular protease production by *Drosophila* imaginal discs. Dev. Biol. 132: 282-291.

Prasad, S.V., Ryan, R.O., Law, J.H., and Wells, M.A. 1986. Changes in lipoprotein composition during larval-pupal metamorphosis of an insect, *Manduca sexta*. J. Biol. Chem. 261: 558-562.
Ramesh, N., Sugumaran, M., and Mole, J.E. 1988. Purification and characterization of two trypsin inhibitors from the hemolymph of *Manduca sexta* larvae. J. Biol. Chem. 263: 11523-11527.
Sasaki, T. 1984. Amino acid sequence of a novel Kunitz-type chymotrypsin inhibitor from hemolymph of silkworm larvae, *Bombyx mori*. FEBS Lett. 168: 227-230.
Sasaki, T. 1985. The reactive site of silkworm hemolymph antichymotrypsin is located at the COOH-terminal region of the molecule. Biochem. Biophys. Res. Commun. 132: 320-326.
Sasaki, T. and Kobayashi, K. 1984. Isolation of two novel proteinase inhibitors from hemolymph of silkworm larva, *Bombyx mori*. Comparison with human serum proteinase inhibitors. J. Biochem. 95: 1009-1017.
Sasaki, T., Kobayashi, K., and Ozeki, T. 1987. Interaction of silkworm larval hemolymph antitrypsin and bovine trypsin. J. Biochem. 102: 433-441.
Saul, S.J. and Sugumaran, M. 1986. Protease inhibitor controls prophenoloxidase activation in *Manduca sexta*. FEBS Lett. 208: 113-116.
Saul, S.J. and Sugumaran, M. 1987. Protease mediated prophenoloxidase activation in the hemolymph of the tobacco hornworm, *Manduca sexta*. Arch. Insect Biochem. Physiol. 5: 1-11.
St. Leger, R.J., Durrands, P.K., Charnley, A.K., and Cooper, R.M. 1988. Role of extracellular chymoelastase in the virulence of Metarhizium anisopliae for *Manduca sexta*. J. Invertebr. Pathol. 52: 285-293.
Yoshida, H. and Ashida, M. 1986. Microbial activation of two serine enzymes and prophenoloxidase in the plasma fraction of hemolymph of the silkworm, *Bombyx mori*. Insect Biochem. 16: 539-545.

BIOSYNTHESIS AND ENDOCYTOSIS OF YOLK PROTEINS IN THE MOSQUITO

Alexander S. Raikhel, Tarlochan S. Dhadialla, Wen-Long Cho,
Alan R. Hays and C. Noah Koller

Department of Entomology and
Programs in Cell and Molecular Biology and Genetics
Michigan State University
East Lansing, Michigan 48824, U.S.A.

INTRODUCTION

The maturation of insect eggs requires the fat body production of large amounts of a protein, vitellogenin (Vg), that is secreted into the hemolymph, selectively accumulated by the oocytes and stored in yolk bodies as crystalline vitellin (Vn). The coordination of activity in fat body and oocytes during vitellogenesis is achieved through a complex regulatory mechanism. The mosquito, *Aedes aegypti*, has proven to be a valuable organism in the study of vitellogenesis.

Previously, the cellular events occurring in the fat body during each cycle of egg maturation in the mosquito were characterized (Raikhel, 1987a). Using immunocytochemistry at the light and electron microscopical levels, the Vg secretory pathway and its termination by the lysosomal system was revealed (Raikhel and Lea, 1983; Raikhel, 1986a; 1986b; 1986c; 1987b). Similar techniques were also used to investigate the Vg internalization pathway and sorting of specific and non-specific proteins in the oocyte (Raikhel, 1984a; 1987b; Raikhel and Lea, 1986; 1987). The juvenile hormone-mediated development of endocytotic organelles in mosquito oocytes, which result in their competence for protein uptake was also shown (Raikhel, 1984b; 1985). In order to study vitellogenesis at the biochemical and molecular levels, a library of monoclonal antibodies (mAB) directed against mosquito Vn and Vg was produced (Raikhel et al., 1986; Raikhel and Lea, 1987). In this report, we summarize our recent results concerning mosquito vitellogenesis.

BIOSYNTHESIS OF MOSQUITO VITELLOGENIN

Identification of the primary translation product and the co- and post-translational modifications that result in a mature Vg molecule, is central to our understanding both the structure-activity relation for Vg and its biosynthetic regulation. Both normal secretion of Vg and its specific endocytosis by oocytes depends upon the correct processing of Vg in the fat body cell. Information on the complete sequence of the co-and post-translational modifications of insect Vg is only available for *Drosophila*, whose yolk polypeptides are different from other insect vitellogenins (Bownes, 1986). We have identified and characterized the main biosynthetic steps for processing of the primary product of translation of mosquito Vg mRNA into mature secreted Vg.

Characterization of a high-molecular weight precursor

Monoclonal antibodies to *A. aegypti* Vn were used to show that both Vg and Vn are composed of a 200 kDa and a 66 kDa subunit (Raikhel and Bose, 1988). Analyses of cell-free translation products (Bose and Raikhel, 1988), pulse-labeling and microsomal separa-

Molecular Insect Science, Edited by
H. H. Hagedorn *et al.*, Plenum Press, New York, 1990

tions demonstrated that the two subunits originate from a common high-molecular weight precursor, and are, thus, the products of the same gene.

This Vg precursor was identified by immunoprecipitation with mABs specific to different Vg subunits. Cell-free translation of fat body poly(A)$^+$ RNA from vitellogenic females produced an immunoprecipitable 224 kDa Vg precursor that increased to 240 kDa in the presence of dog pancreatic microsomal membranes. A precursor with Mr = 250,000, identified as proVg, was immunoprecipitated in microsomal fractions of vitellogenic fat bodies. With pulse labeling, proVg was detected in homogenates of fat bodies from blood-fed mosquitoes only during the first few hours after the initiation of Vg synthesis. A much greater accumulation of proVg was achieved by an *in vitro* stimulation of Vg synthesis in previtellogenic fat bodies cultured with 20-hydroxyecdysone (Fig. 1A). The proVg was glycosylated and to much lesser degree phosphorylated. Treatment of fat bodies with tunicamycin, an inhibitor of N-linked glycosylation, resulted in a precursor which was neither glycosylated nor phosphorylated. Its apparent molecular weight (226,000) was similar to that of the Vg primary translation product obtained by cell-free translation of fat body poly(A)$^+$ RNA. A similar reduction in molecular weights (23 kDa) of the proVg and both the mature Vg subunits combined after digestion with endo-β-N-acetylglucosaminidase (Endo-H) indicated that glycosylation is completed prior to cleavage of proVg (Fig. 1B).

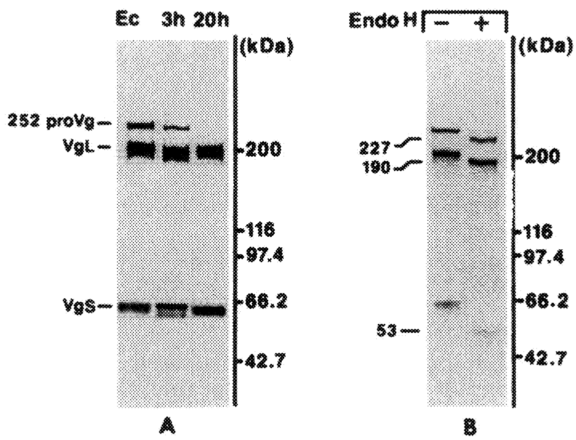


Fig. 1. A. *In vitro* pulse-labeling of mosquito fat bodies of various physiological ages. Fat bodies, from vitellogenic females, 3 or 20 h after a blood meal, or from previtellogenic females, after stimulation with 10^{-5} M 20-hydroxyecdysone for 6 hr *in vitro*, were pulse labeled for two hr with [^{35}S]methionine. Immunoprecipitates of labeled fat body extracts were analyzed by SDS-PAGE and gel fluorography. 252 proVg, processed Vg precursor; VgL, large Vg subunit (200 kDa); VgS, small Vg subunit (66 kDa).

B. Effect of Endo-H on the mosquito Vg precursor and the mature Vg subunits. Fat bodies, stimulated with 20-hydroxyecdysone as above, were pulse-labeled with[^{35}S]methionine. Immunoprecipitates of fat body homogenates were digested with Endo-H for 16 hr at 37^{o} C. After the digestion, the products were analyzed by SDS-PAGE. Presence and absence of Endo-H is indicated by + and -, respectively. The 227, 190 and 53 kDa molecules are the reduced Mr of the 252 proVg, VgL and VgS respectively, after Endo-H digestion (+). The molecular weights on the right in both figures in order of decreasing Mr are myosin, a-galactosidase, phosphorylase b, bovine serum albumin and ovalbumin (Bio-Rad).

Post-translational modifications

In vitro pulse-chase experiments revealed rapid proteolytic cleavage of proVg to two polypeptides with Mr = 190,000 and 62,000 (Fig. 2). Treatment of fat bodies with tunicamycin *in vitro* inhibited glycosylation, phosphorylation and secretion, but not the cleavage of the nascent Vg precursor into 185 kDa and 49 kDa polypeptides. Treatment of

secreted Vg with Endo H and alkaline phosphatase demonstrated that mos of the phosphorylated moieties are located on protein backbone of Vg molecule. Further pulse-chase experiments disclosed that transformation of the cleavage products into mature 200 kDa and 66 kDa Vg subunits occurred as the last step prior to Vg secretion (Fig. 2). The increase in size of both the Vg subunits was inhibited by monensin, an ionophore which disrupts the function of the Golgi complex. Sulfation as an additional, previously unknown, modification of mosquito Vg was shown by the incorporation of sodium [^{35}S]sulfate into both Vg subunits. Since sulfation of Vg was predominantly blocked by monensin, the final maturation of Vg subunits in the Golgi complex is, at least in part, due to this modification.

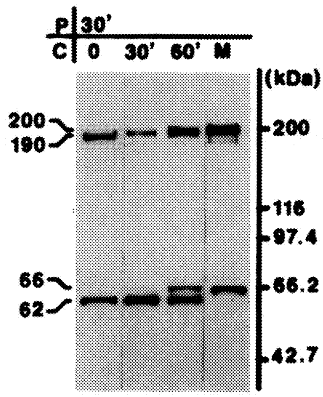


Fig. 2. Time course of [^{35}S]methionine labeling of Vg in mosquito fat bodies. Fat bodies from mosquitoes (18 hr post-blood meal) were pulse labeled with [^{35}S]methionine for 30 min and chased for indicated times in complete medium. Fat body extracts and proteins secreted into the 120 min chase medium (M) were immunoprecipitated and subjected to SDS-PAGE. The molecular weight standards are as in Fig. 1.

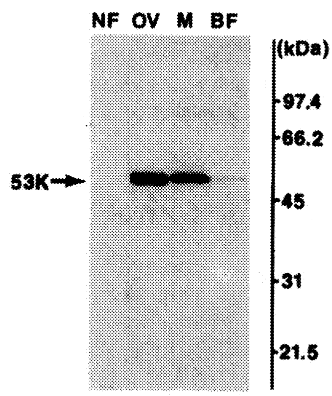


Fig. 3. Immunoblot of 53 kDa protein. Protein extracts from vitellogenic ovaries (OV) and from fat bodies of vitellogenic (BF), non-vitellogenic (NF) female mosquitoes, and media secretion from vitellogenic fat bodies (M) were subjected to SDS-PAGE on a 9-12% gradient gel. Proteins were then transferred onto nitrocellulose membrane and treated with anti-53 kDa polyclonal antibodies. In a separate experiment proteins from the male fat body were also used for immunoblot analysis (not shown). The 53 kDa protein was detected only in fat bodies, ovaries of vitellogenic females and media secretion by vitellogenic fat bodies.

SECRETORY ACTIVITY OF THE FAT BODY: IDENTIFICATION AND CHARACTERIZATION OF A NEW VITELLOGENIC PROTEIN

Analysis of proteins secreted by female fat bodies cultured *in vitro* demonstrated that during vitellogenesis, in addition to Vg, another protein was synthesized, secreted and accumulated in ovaries. This protein has an apparent molecular weight of 53,000 under both reducing and non-reducing conditions. It was purified, by anion exchange chromatography followed by preparative SDS-PAGE, from extracts of vitellogenic ovaries. Polyclonal antibodies to the purified protein were produced in rabbits. Pulse labeling of vitellogenic fat bodies with [^{3}H]mannose and [^{35}S]methionine in the presence or absence of tunicamycin followed by immunoprecipitation demonstrated that the 53 kDa protein was glycosylated and glycosylation accounts for 1.2 kDa of its molecular mass. Antibodies were also used to demonstrate tissue- and stage-specificity (Fig.3) as well as the kinetics of its synthesis during the vitellogenic cycle. The synthesis of the 53 kDa protein by the female fat body begins between 0-4 hr after the initiation of vitellogenesis by a blood meal. It peaks between 18-24 hr and drops to a background level by 48 hr (Fig. 4). Both the synthesis and secretion of the 53 kDa protein could be stimulated by 10^{-6} M 20-hydroxyecdysone in previtellogenic female fat bodies cultured *in vitro*. The properties of this additional sex- and stage-specific protein--its size, kinetics, and regulation of its synthesis--make it useful for investigating hormonal control of expression of fat body genes during vitellogenesis.

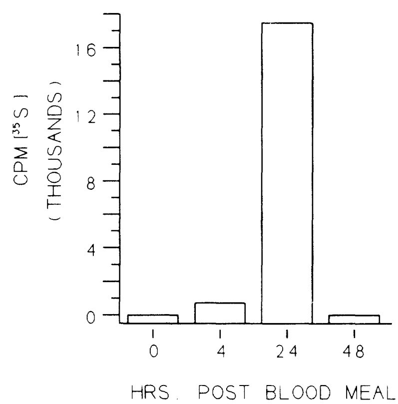


Fig. 4. Time course of secretion of the 53 kDa protein by mosquito vitellogenic fat bodies. Five fat bodies were dissected at the indicated times and incubated for two hr in culture medium containing [^{35}S]-methionine. The 53 kDa protein was immunoprecipitated using polyclonal antibodies and its radioactivity quantified.

CYCLICITY OF VITELLOGENIC ACTIVITIES IN THE FAT BODY

As a consequence of cyclic egg production, Vg synthesis in the mosquito fat body is periodic. During each egg maturation cycle, the fat body cells undergo three successive stages of activity: (1) proliferation of biosynthetic organelles; (2) the synthesis of vitellogenic proteins; and (3) termination of Vg production and breakdown of the biosynthetic organelles by lysosomes (Raikhel and Lea, 1983; Raikhel, 1986a; 1986b).

Proliferation of ribosomes

Previous electron microscopical observations (Raikhel and Lea, 1983) suggested that production of ribosomes first occurs in the fat body of the adult female mosquito after eclosion and then again during the first 12-16 hr after a blood meal. The previtellogenic development is critical for the mosquito fat body to acquire competence for Vg synthesis, but the nature of this development is unclear (Flanagan and Hagedorn, 1977; Ma et al.,

1988). We investigated activity of the fat body cells during this period (Raikhel and Lea, 1990) and found that nucleolus, the organelle responsible for producing ribosomes, enlarges three-fold reaching maximal size between 2 and 3 days after eclosion. The granular component of the nucleolus containing ribosomal precursors increases considerably as well. These signs of nucleolar activation correlate with the synthetic rate and accumulation of poly(A)$^-$ RNA (predominantly ribosomal RNA) in the fat body cells. The amount of poly(A)$^-$ RNA in the fat body cells increases during the first two days after eclosion and then declines gradually. The rate of RNA synthesis exhibited similar kinetics, but with sharper rise and decline than that for poly(A)$^-$ RNA (Fig. 5). All characteristics of nucleolar activation, its enlargement, accumulation of poly(A)$^-$ RNA and the increased rate of RNA synthesis, are blocked by removal of the corpora allata (CA) in newly eclosed adult females but could be restored by either implantation of corpora allata or topical application of juvenile hormone III or its analog, 7-S-methoprene, to allatectomized females (Raikhel and Lea, 1990). Thus, previtellogenic activation of fat body nucleoli for ribosomal RNA pro-

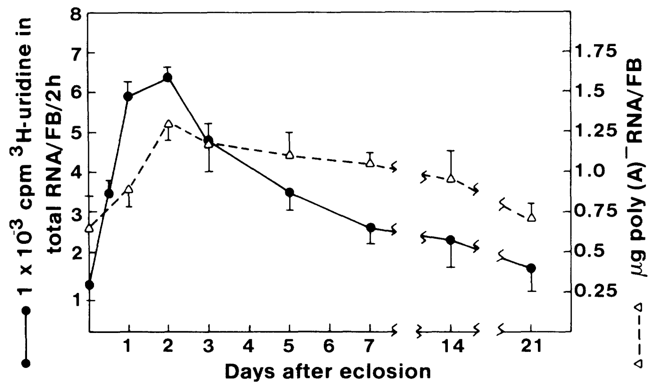


Fig. 5. The rate of RNA synthesis and accumulation of poly(A)$^-$ RNA in the fat body of female mosquitoes during previtellogenic development and the state-of-arrest. For determination of poly(A)$^-$ RNA, three sets of 25 fat bodies were used for each time point. For the estimation of RNA synthetic rates, females were injected with [^{3}H]uridine at different times after eclosion. After two hr the radioactivity in total RNA from the fat bodies was quantified. Three sets of fat bodies were used for each time point. Each point represents the mean ±SEM (from Raikhel and Lea, 1990, with permission).

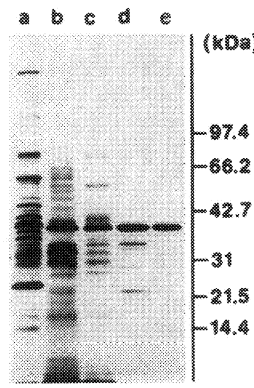


Fig. 6. Analysis of purification of the mosquito cathepsin D by gradient SDS-PAGE (10-15%) as revealed with silver staining. Lanes: (a) soluble proteins from headless previtellogenic mosquitoes; (b) 30-50% ammonium sulfate precipitated proteins from a; (c) supernatant from b after acid precipitation at pH 2.5; (d) pooled fractions (32) containing cathepsin D activity after S-sepharose chromatography; (e) pooled fractions (3) with peak cathepsin D activity before pooling d. The molecular weights on the right in order of decreasing Mr are phorphorylase b, BSA, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme.

duction is controlled by juvenile hormone. Further studies should clarify functions of the previtellogenic fat body which depend upon juvenile hormone-controlled production of ribosomes.

Development of a probe for studying lysosomal activity in the fat body

Lysosomes play an important role in termination of Vg secretion from the mosquito fat body (Raikhel, 1986a; 1986b; 1986c). During the cessation of Vg synthesis, Vg-containing secretory granules are degraded in lysosomes instead of being secreted. Furthermore, by the end of the vitellogenic cycle protein synthetic machinery of fat body cells undergoes lysosomal degradation. As a basis for studying regulation of this lysosomal activity at the molecular level, a lysosomal enzyme, cathepsin D, was purified and characterized (Fig. 6). The native molecular weight of the purified enzyme determined by PAGE under non-denaturing conditions was 80,000. The enzyme was resolved with SDS-PAGE into a single 40 kDa peptide, suggesting that it exists as a dimer in its native state. The pI of the purified enzyme, determined by isoelectric focusing gel electrophoresis, was 5.4. The purified enzyme exhibits characteristics of cathepsin D. It utilizes hemoglobin as a substrate and its activity is completely inhibited by pepstatin-A and 6 M urea but not by 10 mM KCN. Optimal activity of the purified cathepsin was obtained at pH 3.0 and 45°C. Sequencing of 19 amino acid of N-terminal of mosquito cathepsin D shows 74% identity to the N-terminal of porcine cathepsin D and an internal sequence of human cathepsin D.

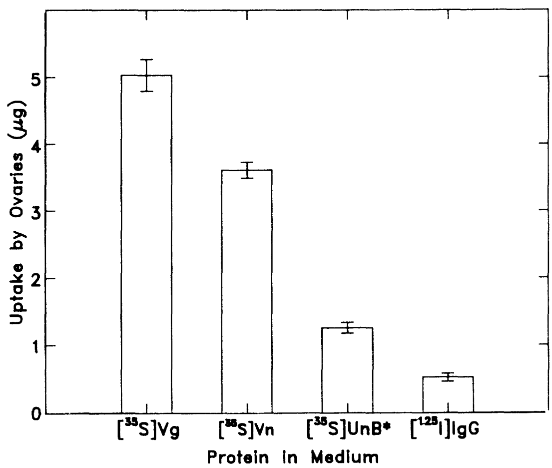


Fig. 7. Uptake of various proteins by single ovaries incubated *in vitro*. Ovaries, 180 µm yolk length (18 hr post-blood meal), were incubated at 27°C for two hr, following a one hr preincubation, in 10 µl of incubation medium containing labeled proteins. Values are the means of three replicates. *This is the combined fraction of proteins, including the 53 kDa protein, labeled in the *in vitro* fat body culture but not bound to DEAE-Sepharose CL-6B.

SELECTIVE ENDOCYTOSIS IN MOSQUITO OOCYTES

Selective accumulation of Vg from the hemolymph by developing oocytes and its subsequent deposition into yolk bodies determines the success of insect egg maturation and, ultimately, the development of an embryo. The developmental synchrony, rapid egg maturation and a stringently controlled threshold of mosquito vitellogenesis, make mosquitoes a good system for the investigation of selective endocytosis in an insect oocyte.

We began with the development of an *in vitro* assay to study the uptake of Vg by mosquito oocytes. Vitellogenin was metabolically labeled to a high specific activity (3 to 6x 10^5 CPM/µg) by incubating vitellogenic fat bodies in the presence of [^{35}S]methionine *in vitro*. Purification of both Vg and Vn from ovaries was achieved with ion-exchange chromatography on DEAE-Sepharose CL-6B. In an optimized *in vitro* system, Vg uptake was maximal at pH 7.5. It declined at basic and acid pH, and ceased abruptly at pH 6.3.

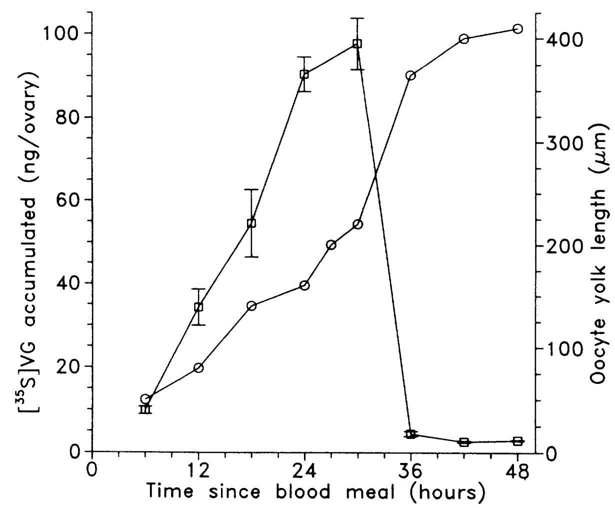


Fig 8. Effect of oocyte developmental stage on accumulation rate of Vg by ovaries (□). The size of oocytes, in ovaries selected at each time indicated, was based on the relation shown between hours post blood meal and yolk length (o). One ovary was incubated in wells with 10 μl incubation medium and 0.18 μg Vg/μl (6.77 x 10^5 CPM/μg). Incubation proceeded for two hr after a one hr preincubation period. (From Koller et al, 1989, with permission).

Accumulation of Vg by mosquito oocytes demonstrated properties of receptor-mediated endocytosis; i.e. temperature dependence, saturability, selectivity, and tissue specificity. The uptake of Vg was inhibited at 4°C with only 6% of the uptake achieved at 27°C. At 24 hr post-blood meal, uptake of Vg by oocytes at 27°C, neared saturation with a Vg concentration in the medium of 8 μg/μl. Saturation kinetics generated for Vg by these oocytes produced a V_{max} = 3.2 μg/μl/hr and an apparent $K_{uptake} = 8.4 \times 10^{-6}$ M. Ovaries accumulated 10 times the amount of Vg compared to mouse IgG. Uptake of Vn was 72% of that for Vg, indicating that oocytes distinguished these proteins. The unbound fraction from ion-exchange chromatography of secreted fat body proteins, including the 53 kDa protein, was taken up at 25% of the rate for Vg. This indicated the possibility of specific uptake of some proteins from this mixture (Fig. 7). Non-ovarian tissues, fat body and Malpighian tubules, accumulated both Vg and IgG at equally low levels. During the vitellogenic cycle, the rate of Vg uptake by oocytes showed a steep and linear increase between six and 24 hr post-blood meal. The peak of Vg uptake occurs between 24 and 30 hr post-blood meal, followed by a precipitous decline and cessation of uptake by 36 hr (Fig. 8). The developed *in vitro* assay for Vg uptake is now being used for studying regulation of endocytotic activity of mosquito oocytes.

ACKNOWLEDGMENTS.

This work was supported by grants AI-24716 and HD-22958 from the National Institutes of Health to ASR.

REFERENCES

Bose, S. G. and Raikhel, A. S. 1988. Mosquito vitellogenin subunits originate from a common precursor. Biochem. Biophys. Res. Comm. 155: 436-442.

Bownes, M. 1986. Expression of the genes coding for vitellogenin (yolk protein). Ann. Rev. Entomol. 31: 507-531.

Flanagan, T. R. and Hagedorn, H. H. 1977. Vitellogenin synthesis in the mosquito: the role of juvenile hormone in the development of responsiveness to ecdysone. Physiol. Ent. 2: 173-178.

Koller, C. N., Dhadialla, T. S. and Raikhel, A. S. 1989 Selective endocytosis of vitellogenin

by oocytes of the mosquito *Aedes aegypti*: an *in vitro* study. Insect Biochem. 19: 693-702.

Ma, M., Zhang, J.-Z., Gong, H., and Gwadz, R. 1988. Permissive action of juvenile hormone on vitellogenin production by the mosquito, *Aedes aegypti*. J. Insect Physiol. 34: 593-596.

Raikhel, A. S. 1984a. The accumulative pathway of vitellogenin in the mosquito oocyte; a high resolution immuno- and cytochemical study. J. Ultrastr. Res. 87: 285-302.

Raikhel, A. S. 1984b. Accumulation of membrane-free clathrin-like lattices in the mosquito oocyte. Euro. J. Cell. 35: 279-283.

Raikhel, A. S. 1986a. Role of lysosomes in regulating of vitellogenin secretion in the mosquito fat body. J. Insect Physiol. 32: 597-604.

Raikhel, A. S. 1986b. Lysosomes in the cessation of vitellogenin secretion by the mosquito fat body: Selective degradation of Golgi complexes and secretory granules. Tissue and Cell 18: 125-142.

Raikhel, A. S. 1986c. Lysosomal activity in the fat body - a novel mechanism involved in the termination of mosquito vitellogenesis. pp 25-31 in: "Host Regulated Developmental Mechanisms in Vector Arthropods." D. Borovsky and A. Spielman, eds. University of Florida, IFAS, Florida Medical Entomology Laboratory.

Raikhel, A. S. 1987a. The cell biology of mosquito vitellogenesis. Memorias do Instituto Oswaldo Cruz 82, suppl. III: 93-101.

Raikhel, A. S. 1987b. Monoclonal antibodies as probes for processing of yolk protein in the mosquito; a high-resolution immunolocalization of secretory and accumulative pathways. Tissue and Cell 19: 515-529.

Raikhel, A. S. and Bose, S. G. 1988. Properties of mosquito yolk protein: A study using monoclonal antibodies. Insect Biochem. 18: 565-575.

Raikhel, A. S. and A. O. Lea. 1983. Previtellogenic development and vitellogenin synthesis in the fat body of a mosquito: An ultrastructural and immunocytochemical study. Tissue Cell 15: 281-300.

Raikhel, A. S. and A. O. Lea. 1985. Hormone-mediated formation of the endocytic complex in mosquito oocytes. Gen. Comp. Endocrinol. 53: 424-435.

Raikhel, A. S. and Lea A. O. 1986. The specific ligand, vitellogenin, directs internalized proteins into accumulative compartments of mosquito oocytes. Tissue Cell 18:559-574.

Raikhel, A. S. and Lea, A. O. 1987. Analysis of mosquito yolk protein by monoclonal antibodies. pp 403-413 in: "Molecular Entomology. UCLA Symposia on Molecular and Cellular Biology, New Series." Vol. 49. J. Law, ed. Alan R. Liss, Inc., New York.

Raikhel, A. S. and Lea A. O. 1990. Juvenile hormone controls previtellogenic proliferation of ribosomal RNA in the mosquito fat body. Gen. Comp. Endocrinol. 77: 423-434.

Raikhel, A. S., Pratt, L., and Lea A. O. 1986. Monoclonal antibodies as probes for processing of yolk protein in the mosquito; production and characterization. J. Insect Physiol. 32: 879-890.

MOLECULAR PROPERTIES, FUNCTIONS AND DEVELOPMENTALLY REGULATED BIOSYNTHESIS OF ARYLPHORIN IN *Calliphora vicina*

Klaus Scheller, Beate Fischer, and Heide Schenkel[1]

Institute of Zoology, University of Würzburg,
Röntgenring 10, D-8700 Würzburg, Germany.
[1]present address: Institute of Zoology, University of Heidelberg

INTRODUCTION

The pioneering work of Munn and colleagues (Munn and Greville, 1969) was the first tangible indication that the larvae of holometabolous insects synthesize large amounts of unusual proteins which accumulate in their hemolymph. These proteins, generally referred to as larval serum proteins (LSPs) or storage proteins, have many common characteristics. They are synthesized by the fat body of actively feeding larvae and their concentrations increase enormously in the last larval instar, making up the major component of the whole larval soluble proteins. They form hexamers in the 5×10^5 Dalton range and dissociate into polypeptides of $7.2 - 9 \times 10^4$ Daltons (For review, see Levenbook, 1985). Telfer et al. (1983) suggested that the larval haemolymph proteins which are similar in structure and amino acid composition to calliphorin, the major haemolymph protein of the blowfly, *Calliphora vicina*, should be called arylphorins to signify that they bear aryl groups.

Purified *Calliphora* arylphorin is a lipo-glycoprotein composed of a series of closely related but heterogeneous hexamers of Mr ca. 500 kDa. It reversibly dissociates into its ca. 80 kDa constituent subunits at pH values >6.5. At pH 9, up to 9 polypeptides can be resolved by denaturing polyacrylamide gel electrophoresis. Under the conditions prevailing in larval haemolymph (pH 7.0 - 7.1, an osmolarity equivalent to 0.15 M KCl, and an arylphorin concentration of about 10% (w/v)) the protein undoubtedly exists as a hexamer (Levenbook, 1983). Palli and Locke (1987) propose a triangular antiprism structure with six subunits arranged at the vertices of an octahedron. A table summarizing the molecular properties of different arylphorins is given by Peter and Scheller (1989).

Arylphorins contain carbohydrate and phospholipid residues. The arylphorin of *Musca domestica* has glycosylated and unglycosylated subunits in the hexameric complex (Marinotti et al. 1988). The carbohydrate structure of *Manduca sexta* arylphorin was shown by NMR spectroscopy to consist of the triantennary oligosaccharide Man9GlcNac2 (Ryan et al., 1985). Preliminary data indicated that two different oligosaccharides, probably N-glycanes of the "high-mannose-type" are present in *Calliphora* arylphorin (M.G. Peter, personal communication) with a total carbohydrate content of 0.4% - 0.5%.

Another feature of the arylphorins is the unusually high content of aromatic amino acids and the very low content of cysteine. Tyrosine accounts for 12.2 mol %, and phenylalanine for 11.1 mol % in *Calliphora* arylphorin. Dipteran arylphorin appears to have the highest proportion of tyrosine of any protein whose amino acid composition is known in addition to papain (14.7 mol % tyrosine) and silk fibroin. This fact leads to a considerable overestimation of the protein content using the common method of Lowry et al. (1951).

Polyclonal antisera raised against *Calliphora* arylphorin cross-react with arylphorins of many dipteran species, e.g. *Ceratitis capitata, Dacus oleae, Drosophila melanogaster, Lucilia cuprina, M. domestica, and Sarcophaga peregrina* (unpublished observations). Likewise, the lepidopteran arylphorins from *Bombyx mori, Hyalophora cecropia, and M.*

sexta, are immunologically related; however, they show no cross-reactivity with dipteran arylphorins (Riddiford and Law, 1983).

Organization and Evolution of the Genes Encoding Arylphorin

Because of their relatedness amongst the insects and the stage- and tissue-specific regulation of their expression during development, the arylphorins provide an attractive model system for a study of the evolution of a gene system at both the structural and the regulatory level. Therefore, we decided to characterize the gene encoding arylphorin in *C. vicina* in order to investigate the mechanisms controlling its expression.

A recombinant genomic library of *C. vicina* was constructed by inserting *Sau*3A restricted DNA into a EMBL3 vector and screened with radioactively labeled poly(A)$^+$ RNA from fat bodies of 3rd instar feeding larvae (Schenkel et al., 1985). Five recombinant phages were randomly chosen for a detailed analysis. Evidence that these phages contained arylphorin encoding sequences was obtained by selection of the complementary RNA and its *in vitro* translation. The hybrid-selected RNA directed the translation of an 80 kDa polypeptide which was immunologically identified as arylphorin. Furthermore, the restriction maps of the DNA inserts were established and some DNA inserts were selected for sequencing. The results can be summarized as follows:

1. Each of the recombinant phages tested contains at least 2 coding sequences which are tandemely arranged in direct repeat orientation (Fig. 2a).
2. The coding region is interrupted by one small intron and displays a size of 2.4 kb. This length corresponds to a protein coding capacity of 80 - 85 kDa, the size of the arylphorin subunit.
3. The restriction patterns of the coding regions appear to be slightly different. Examination of heteroduplexes formed between 2 plasmids containing 2 different arylphorin genes confirms the extensive homology of the two genes over a 2.3 kb length of DNA.
4. The coding sequences are separated by intergenic DNA of variable length (3.5 - 5.2 kbp) and different restriction patterns. The intergenic sequences are supposed to contain regulatory sequences.
5. By means of *in situ* hybridization to polytene chromosomes of trichogenic cells of pharate adult pupae of *C. vicina* the arylphorin encoding sequences could be identified as a gene cluster on chromosome 2, near the puff site 4a.

These data are strongly suggestive that the closely related genes belong to a large arylphorin multigene family.

Further experiments confirm the existence of a low degree of polymorphism of the arylphorin genes even in individual adults in pure inbred lines (Schenkel and Scheller, 1986). The arylphorin gene family is not subjected to major rearrangements or selective amplification of some repeats during development. The DNA from the highly polyploid larval fat body cells shows the same restriction pattern like DNA prepared from embryos, adults, larval brains, or wing disks.

Multiply linked arylphorin genes may also exist in *L. cuprina* and *S. peregrina*, which are both closely related to *C. vicina,* (Thomson et al., 1976; Tahara et al., 1984). This type of organization, multiplicity and clustering of genes, differs sharply from that of the arylphorin genes in *D. melanogaster* where single gene copies for the subunits LSP-1α, β and γ residing on different chromosomal sites (Lepesant et al, 1982) have been described. Furthermore, in *B. mori* (Fujii et al., 1989) as well as in *Galleria mellonella*(Ray et al., 1987b) and *M. sexta* (Willott et al., 1989) single copy genes code for arylphorin.

From immunological studies of arylphorins of different insects and comparison of the amino acid and DNA sequences it has been suggested that the genes encoding the arylphorin subunits derive from a common ancestor. The mechanisms which lead to relationships between single copy genes as well as between members of a gene family are exemplarily discussed by Roberts (1983) for the *Drosophila* arylphorins.

Comparing the first exons of the arylphorin genes of *Calliphora* and *Drosophila* we find a considerable degree of homology as depicted in Table 1. In the primary structure of the 4 compared genes, the positively charged amino terminus (Met-Lys) is followed by a cluster of 14-16 hydrophobic amino acids, indicating the occurrence of a typical "signal peptide" for transmembrane secretion in nascent arylphorin. A very similar organization of the first exon was also detected in *Bombyx* (Fujii et al., 1989) and *Manduca* arylphorin (Willot et al., 1989).

Recent data suggest that arylphorins are derived from a hemocyanin archetype. Hemocyanin, a blue copper-protein in the haemolymph of arachnids, xiphosurans, centipedes, and the higher crustaceans, is constructed from copies of 8 different polypeptides which are arranged as cubic hexamers or multiples of hexamers. From experiments published by Markl and Winter (1989) it may be supposed that the spatial arrangement of the entire amino acid backbone of hemocyanin may also fit the primary structure of arylyphorins. Primary structural evidence has also demonstrated arthropod hemocyanin to be related to *Drosophila* arylphorin LSP-2 (Kejzlerová-Lepesant et al., 1987). It is worth mentioning that the immunological properties of arylphorins are useful for the study of phylogenetic relationships as shown for the Tephritidae family. (Kefaliakou et al., 1987).

Table 1: Deduced amino acid sequence encoded by the first exon of the *Calliphora* arylphorin genomic clone pC223 (cal) (see Fig. 2). Degree of homology compared to 3 *Drosophila melanogaster* clones LSP-1α, β, and γ (Delaney et al., 1986): cal/α - 64%; cal/β - 67%; cal/γ - 69%; a/ß - 82%; α/γ - 65%; β/γ - 65%.

```
         10          20           30
MKIAIALLAI  VGLG--ASIT  -DKKVKIADK

         40          50           60
DFLAKQKFLF  EIVYRVENPL  MFEEWNKMGK

         70
FFIFNKADYN
```

Underlined amino acids are identical in cal and two LSPs.
Printed in outlined letters: Amino acids identical in all 4 genes.

Stage- and tissue specific biosynthesis of arylphorin

From immunoblot analyses it has become evident that arylphorin is ubiquitous in all tissues from the 1st larval instar of Calliphora up to adult stage. By contrast, in freshly deposited eggs as well as in late embryos, arylphorin could not be detected. In view of this observation we asked whether the protein was synthesized in different tissues or derives from a common source. Numerous Northern analyses demonstrated that the transcription of the arylphorin gene family is restricted to fat body cells. Therefore, we analyzed the biosynthesis of arylphorin in the fat body with particular interest in stage- and tissue-specific transcription and translation of arylphorin mRNA. The results are shown in Fig.1

In this context it is necessary to mention that in lepidopteran insects the synthesis of arylphorins is not limited to the fat body. In *Calpodes ethlius*, some arylphorin is synthesized in other tissues such as the epidermis, midgut, pericardial cells, and hemocytes. (Leung et al., 1989). In *M. sexta* translatable arylphorin mRNA has been extracted from larval epidermis (Riddiford and Hice, 1985).

To answer the question whether arylphorin mRNA is always translated when it is present in the fat body we had to evaluate the time course of arylphorin biosynthesis *in vivo*. Fig. 1 demonstrates that arylphorin is not synthesized in wandering larvae, puparia and pupae, although arylphorin mRNA is present as shown by Northern analyses and by *in vitro* translation experiments (Sekeris and Scheller, 1977). We know from earlier experiments (Schenkel et al., 1983) that in wandering larvae arylphorin mRNA is kept in shape of non-translated mRNPs which are rapidly degraded at the beginning of pupariation. Similarly in *D. melanogaster* a translational switch inhibits arylphorin mRNA during the hours preceding puparium formation in spite of the presence of considerable amounts of messenger RNA (Powell et al., 1984).

To clarify the question, whether arylphorin genes are also transcribed during the wandering stage when the arylphorin mRNA is not translated into protein, in vivo labeled poly(A)$^+$ RNA from different developmental stages was hybridized with filter-bound arylphorin DNA. The results, shown in Fig. 1, demonstrate that de novo synthesis of arylphorin

mRNA lasts after the feeding stage but continuously decreases during the wandering period and stops at the beginning of pupariation.

We therefore conclude that the tissue-specific biosynthesis of arylphorin is developmentally regulated in the following way: While transcription of arylphorin mRNA is paralleled by its translation in young, rapidly growing larvae, translation stops in late larvae which are ready for metamorphosis, although arylphorin mRNA is still present and transcription of the arylphorin genes continues. That means, the biosynthesis of arylphorin in *C. vinina* is regulated not only at the transcriptional level. Nevertheless, it is interesting to learn more about the mechanisms which control the stage- and tissue specific activation and inactivation of the arylphorin genes.

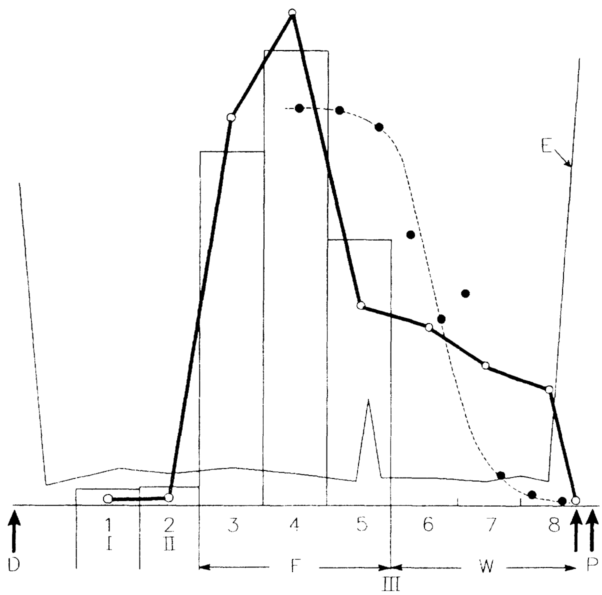


Fig. 1. Arylphorin biosynthesis in the fat body during larval development of Calliphora vicina. Abscissa: ages of animals in days after hatching. D = egg deposition. I-III = larval instars. W = wandering. P = pupation. E = Ecdysteroid titre in the haemolymph (adapted from Koolman, 1980). Arylphorin biosynthesis calculated from *in vivo* [^{35}S] -methionine labeled immunoprecipitates of fat body proteins with anti-arylphorin-IgGs. o----o Relative amount of arylphorin mRNA estimated from Northern blot analyses. o----o Transcription rate of arylphorin genes. Hybridization of in vivo [^{3}H]-uridine labeled poly (A)$^{+}$ RNA with the arylphorin gene pC223 (See Fig. 2.).

We have determined the nucleotide sequence at the 5'-end (pC 209) of the arylphorin gene clone pC223 (Fig. 2b), because it is well established that the cis-acting transcriptional elements for spatial and developmental regulation of gene expression are located around the start site of transcription. A TATA-box, essential for correct transcription, is present at nearly the same position as in the arylphorin genes of *D. melanogaster* (Delaney et al., 1986) and *B. mori* (Fujii et al., 1989). A CAT-box, like in *S. peregrina* and *B. mori* is lacking. A palindromic structure including the GCN4 sequence of Saccharomyces is located at position -502. The product of the GCN4 gene is necessary in yeast for the transcriptional induction of many amino acid bio-synthetic enzymes in response to lack of amino acids (Struhl, 1987). It is tempting to connect the presence of that cis-regulatory element with the transition from feeding to the post-feeding stage when a series of genes is switched on in the fat body of *C. vinina* (Mascheck et al., 1977).

Although essentially the same developmental profiles of arylphorin synthesis were observed in a variety of holometabolous insects the proximate cues either for activation and inactivation of the genes or the cessation of translation have not been understood. From experiments involving the temperature-sensitive *D. melanogaster* mutant ecd^{1}, which becomes deficient in ecdysone after a temperature shift from 20°C to 29°C, it has been sug-

gested that ecdysteroids might be involved in the enhancement of the arylphorin gene activation (Lepesant et al., 1982). In *C. vicina*, a relation between ecdysteroid hormones and the initiation of the transcription of arylphorin genes cannot be stated. Inspection of the 5'-flanking sequence contained in pC209 of the *C. vicina* arylphorin gene (Fig. 2b) give no indication for the presence of an ecdysteroid response element such as is seen in *D. melanogaster* (Riddibough and Pelham, 1987). However, cessation of arylphorin synthesis and degradation of arylphorin mRNA (Schenkel et al., 1983) is concomitant with a rise in the ecdysteroid titre (Fig. 1). Application of exogenous 20-hydroxyecdysone causes a decline of arylphorin mRNA in *C. vicina* (unpublished observations) as well as in *G. mellonella* (Ray et al., 1987a) and *M. sexta* (Webb and Riddiford, 1988) indicating a posttranscriptionally regulated shut off of the arylphorin biosynthesis.

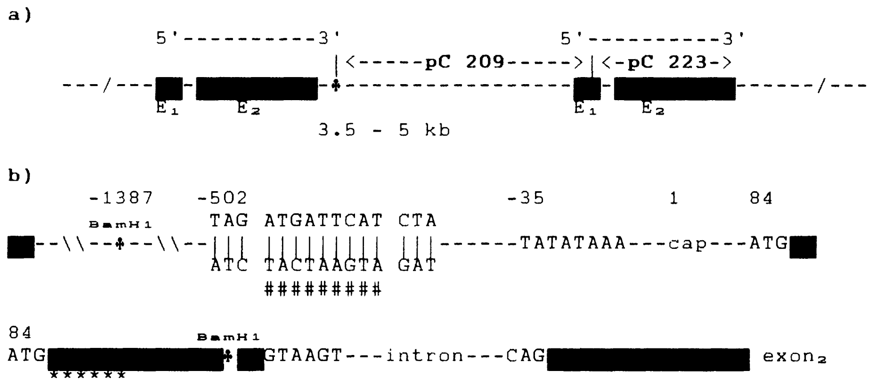


Fig. 2.a) Tandem arrangement of arylphorin genes cloned in EMBL3.
b) Transcription regulatory sequences in the 5'-flanking region pC209 of the arylphorin encloding sequence pC223.
**** Signal peptide sequence; #### GCN4 sequence.

The influence of juvenile hormone on arylphorin biosynthesis is discussed by several authors. It can be suggested that juvenile hormone may block the expression of the genes (Ray et al,. 1987a; Bosquet et al., 1989; Fujii et al., 1989). However a direct regulatory role of juvenile hormone has not yet been presented. In newly moulted Calliphora 3rd instar larvae when the juvenile hormone titre is at the maximum level of 13.3 "Galleria-units"/g body weight (M.I. Bogus, personal communication) transcription of the arylphorin genes is tremendously enhanced. An understanding of the processes regulating arylphorin gene expression will require the characterization of cis-regulatory elements and their interaction with purified nuclear proteins. Experiments to isolate trans-activating factors are presently being done in our laboratory.

Arylphorins as storage proteins

During larval-pupal transformation the fat body cells decrease their role in intermediary metabolism and function mainly to store reserve material for adult tissue formation. Towards the end of the last larval instar arylphorin is reabsorbed from the haemolymph by the fat body. The uptake of arylphorin in *S. peregrina* and *C. vicina* is mediated by a membrane-bound receptor which is activated by 20-hydroxyecdysone (Ueno et al., 1987). Arylphorin is localized in cytoplasmatic granules (Tojo et al., 1980) as well as in the Golgi complex and secretory vesicles (Leung et al., 1989). It is suggested that a large portion of the adult protein is formed de novo from free amino acids or oligopeptides released by the hydrolysis of larval arylphorin, which is supposed to serve as a source of aromatic amino acids (Levenbook, 1983, 1985). However, another way for arylphorins to be utilized could be their incorporation into insoluble material, (ie. as a part of the sclerotized cuticle).

Arylphorin as a constituent of the sclerotized cuticle

Sclerotization of the exoskeleton is an essential process in insect metamorphosis in which 1,2 diphenolic substrates are oxidized by tyrosinase to form nascent quinones in the exocuticle. The diphenolic substrates are ultimately derived from tyrosine. None of the functions of arylphorin, such as a storage protein or ecdysteroid carrier (see below) would require a protein with an exceptionally high content of tyrosine. It has been suggested repeatedly that arylphorins are involved in cuticle formation and sclerotization. Confirming this idea, participation of arylphorins in cuticle sclerotization has been demonstrated using the following approaches (for review, see Peter and Scheller, 1989):

1). Determination of the metabolic fate of radiolabeled arylphorin.
2). Autoradiographic and immunochemical evidence for the presence of arylphorin in the cuticle and sclerotized structures like tracheae.
3). Binding studies with arylphorins and other biopolymers like chitin.
4). Oxidative cross-linking *in vitro* of arylphorin by diphenolic tanning reagents and tyrosinase.

Taking all evidence together, there is little doubt that in Diptera arylphorins are absorbed from the haemolymph by the epidermal cells and secreted in those cuticles which are determined for sclerotization.

Arylphorins as hormone carriers

Steroid hormones circulate in the blood of lower and higher vertebrates partly bound to serum proteins which serve as hormone carriers. Some of these proteins, like transcortin or the sex-homone binding globulin, are found in low concentrations and are characterized by a high specificity and high affinity for special steroids. Other more abundant proteins, especially albumin, reveal a low specificity and low affinity for steroids. There is, so far as we are aware, no unambiguous evidence for the general occurence of serum proteins in insects which serve as steroid carriers. By three independent methods, photoaffinity labelling, equilibrium dialysis, and fluorescence quenching it has been shown that arylphorin in the haemolymph of *C. vicina* binds to ecdysteroids with low affinity (K_d between 3×10^{-5} and 2.5×10^{-3} M) (Enderle et al., 1983). The low affinity is compensated by an exceedingly high arylphorin concentration. During the whole 3rd instar of *C. vicina*, 87% - 90% of ecdysteroid hormones circulating in the haemolymph are bound to arylphorin. Just at the beginning of pupariation, the hormone is released (only 8% remain bound). At the same time, the ecdysteroid titer in the haemolymph rises 6-fold. Preliminary data from our laboratory indicate that the signal responsible for the separation of the hormone from the carrier might be the phosphorylation of a serine residue of arylphorin.

ACKNOWLEDGMENTS

We thank Anneliese Striewe-Conz for competent technical assistance. Research in the authors' laboratory was supported by the Deutsche Forschungsgemeinschaft (Sche 195/4) and the Universitätsbund Würzburg.

REFERENCES

Bosquet, G., Guillet, C., Calvez, B. and Chavancy, G. 1989. The regulation of major haemolymph protein synthesis: Changes in mRNA content during the development of *Bombyx mori* larvae. Insect Biochem. 19: 29-39.

Delaney, S.J., Smith, D.F., McClelland, Sunkel, C. and Glover, D.M. 1986. Sequence conservation around the 5' ends of the larval serum protein 1 genes of *Drosophila melanogaster*. J. Mol. Biol. 189: 1-11.

Enderle, U., Kuser, G. Reum, L., Scheller, K. and Koolman, J. 1983. Ecdysteroids in the haemolymph of blowfly larvae are bound to calliphorin. pp. 40-49 in: "The Larval Serum Proteins of Insects." Scheller, K. ed. Thieme, Stuttgart.

Fujii, T., Sakurai, H., Izumi, S. and Tomino, S. 1989. Structure of the gene for the arylphorin-type storage protein SP 2 of *Bombyx mori*. The J. Biol. Chem. 264: 11020-11025.

Kejzlarov-Lepesant, J., Mousseron, S., Benes, H., Jowett, T., Chihara, C., Claverie, J.-M. and Lepesant, J.-A. 1987. Structure and expression of the LSP-2 gene of *Drosophila melanogaster*. Biol. Chem. Hoppe-Seyler 368: 575-576.

Kefaliakou, M., Christodoulou, C. and Marmaras V.J. 1987. The use of immunological properties of larval serum proteins for the study of phylogenesis in Diptera. Biol. Chem. Hoppe-Seyler 368: 574.

Koolman, J. 1980. Ecdystroids in the blowfly, *Calliphora vicina*. pp. 187-209 in: "Progress in Ecdysone Research." Hoffmann, J. A. ed. Elsevier/North Holland, New York

Lepesant, J.-A., Levine, M., Garen, A., Kejzlerov-Lepesant, J., Rat, L. and Somme-Martin, G. 1982. Developmentally regulated gene expression in *Drosophila* larval fat body. J. Molec. Appl. Gen. 1: 371-383.

Leung, H., Palli, S.R and Locke, M. 1989. The localization of arylphorin in an insect, *Calpodes ethlius*. J. Insect Physiol. 35: 223-231.

Levenbook, L. 1983. The structure and function of calliphorin. pp. 1-17 in: "The Larval Serum Proteins of Insects." Scheller, K. ed. Thieme, Stuttgart.

Levenbook, L. 1985. Insect storage proteins. pp. 307-346 in:"Comprehensive Insect Physiology, Biochemistry and Pharmacology." Vol.10. Kerkut, G.A. and Gilbert, L.I., eds. Pergamon Press, Oxford.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. 1957. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265-275.

Marinotti, O., Nunes, L.R. and de Bianchi, A.G. 1988. Heterogeneous glycosylation of *Musca domestica* arylphorin. Biochem. Biophys. Res. Commun. 151: 1004-1010.

Markl, J. and Winter S. 1989. Sububit-specific monoclonal antibodies to tarantula hemocyanin, and a common epitope shared with calliphorin. J. Comp. Physiol. B. 159: 139-151

Mascheck, P., Scheller, K. and Karlson, P. 1977. Changes in gene expression during the larval development of *Calliphora vicina* induced by ecdysterone. Z. Naturforsch. 32c: 434-438.

Munn, E,.A. and Greville, G.D. 1969. The soluble proteins of developing *Calliphora erythrocephala*, particularly calliphorin, and similar proteins in other insects. J. Insect Physiol. 15: 1935-1950.

Palli, S.R. and Locke, M. 1987. Purification and localization of the three major haemolymph proteins of an insect, *Calpodes ethlius*. Arch. Insect Biochem. Biophys. 5: 233-245.

Peter, M.G. and Scheller, K. 1989. Arylphorins and the integument. in: "The Physiology of Insect Epidermis." Retnakaran, A. and Binnington, K., eds. Inkata Press, North Clayton. in press.

Powell, D., Sato, J.D., Brock, H.W. and Roberts, D.B. 1984. Regulation of synthesis of the larval serum proteins of *Drosophila melanogaster*. Dev. Biol. 102: 206-215.

Ray, A., Memmel, N.A. and Kumaran, A.K. 1987a. Developmental regulation of the larval haemolymph protein genes in *Galleria mellonella*. Roux's Arch. Dev. Biol. 196: 414-420.

Ray, A., Memmel, N.A. Orchekowski, R.P. and Kumaran A.K. 1987b. Isolation of two cDNA clones coding for larval haemolymph proteins of *Galleria mellonella*. Insect Biochem. 17: 603-617.

Riddihough, G. and Pelham, H.R.B. 1987. An ecdysone response element in the *Drosophila* hsp27 promotor. EMBO J. 6: 3729-3734.

Riddiford L.M. and Law J.H. 1983. Larval serum proteins of Lepidoptera. pp. 75-85 in: "The Larval Serum Proteins of Insects." Scheller, K., ed. Thieme, Stuttgart.

Riddiford, L.M. and Hice, R.H. 1985. Developmental profiles of the mRNAs for *Manduca* arylphorin and two other storage proteins during the final larval instar of *Manduca sexta*. Insect Biochem. 15: 489-502.

Roberts, D.B. 1983. The evolution of the larval serun protein genes in *Drosophila*. pp.86-101 in: "The Larval Serum Proteins of Insects." Scheller, K., ed. Thieme, Stuttgart.

Ryan, R.O., Anderson D.R., Grimes W.J. and Law J.H. 1985. Arylphorin from *Manduca sexta*: Carbohydrate structure and immunological studies. Arch. Biochem. Biophys. 243: 115-124.

Schenkel, H., Kejzlarov-Lepesant, J., Berreuer, P., Moreau, J., Scheller, K., Brégégère, F. and Lepesant, J.-A. 1985. Identification and molecular analysis of a multigene family encoding calliphorin, the major larval serum protein of *Calliphora vicina*. EMBO J. 4: 2983-2990.

Schenkel, H., Myllek, C., Konig, M., Hausberg, P. and Scheller, K. 1983. Calliphorin: studies on its biosynthesis and function. pp. 18-39 in: "The Larval Serum Proteins of Insects." Scheller, K., ed. Thieme, Stuttgart.

Schenkel, H.. and Scheller, K. 1986. Stage- and tissue-specific expression of the genes encoding calliphorin, the major larval serum protein of *Calliphora vicina*. Roux's Arch. Dev. Biol. 195: 290-295.

Sekeris, C.E. and Scheller, K. 1977. Calliphorin, a major protein of the blowfly. Correlation between the amount of protein, its biosynthesis, and the titre of translatable calliphorin-mRNA during development. Dev. Biol. 59: 12-23.

Struhl, K. 1987. Promotors, activator proteins and the mechanism of transcriptional initiation in yeast. Cell 49: 295-297.

Tahara, T., Kuroiwa, A., Obinata, M. and Natori, S. 1984. Multi-gene structure of the storage protein genes of *Sarcophaga peregrina*. J. Mol. Biol. 174: 19-29.

Telfer, W.H., Keim, P.S. and Law, J.H. 1983. Arylphorin, a new protein from *Hyalophora cecropia*: Comparisons with calliphorin and manducin. Insect. Biochem. 13: 601-613.

Thomson, J.A., Radok, K.-R., Shaw, D.C., Whitten, M.J., Foster, G.G. and Birt, L.M. 1976. Genetics of lucilin, a storage protein from the sheep blowfly, *Lucilia cuprina*. Biochem. Genet. 14: 145-160.

Tojo, S., Nagata, M. and Kobayashi, M. 1980. Storage proteins in the silkworm, *Bombyx mori*. Insect Biochem. 10: 289-303.

Ueno, K. and Natori, S. 1987. Possible involvement of lumichrome in the binding of storage protein to its receptor in *Sarcophaga peregrina*. J. Biol. Chem. 262: 12780-12784.

Webb, B.A. and Riddiford, L.A. 1988. Regulation of expression of arylphorin and female-specific protein mRNAs in the tobacco hornworm, *Manduca sexta*. Developmental Biology 130: 682-692.

Willott, E., Wang X-Y. and Wells, M.A. 1989. C-DNA and gene sequence of *Manduca sexta* arylphorin: Homology to arthropod hemocyanins. J. Biol. Chem. 264: 19052-19059.

DEVELOPMENTAL AND JUVENILE HORMONE CONTROL OF GENE EXPRESSION IN LOCUST FAT BODY

G.R. Wyatt

Department of Biology
Queen's University
Kingston, Ontario K7L 3N6, Canada

INTRODUCTION

The expression of particular genes in multicellular eukaryotes may be constrained by specificity for tissue, developmental stage, sex and modulation by extracellular factors. In insects, tissue specificity is apparent as in other animal groups, while stage specificity is accentuated by the radical developmental events of molting and metamorphosis. The mechanisms used for sex limitation of gene expression in insects (assuming that we can generalize from the *Drosophila melanogaster* model), involve a cascade of sex-specific transcript splicing (Baker, 1989), and differ profoundly from the vertebrate mechanisms which depend on sex-specific hormones. In differentiated cells, as a result of these factors, genes may either be repressed or exist in an expressible state. In the latter case, they may be expressed either constitutively or subject to modulation by factors such as hormones. In insect development, while coordination is effected by a complex of neurohormones, the immediate modulation of the gene activities is largely the function of the two types of lipoidal hormones, ecdysones and juvenile hormones (JHs).

FAT BODY, JH AND JUVENOID ACTION, AND VITELLOGENIN SYNTHESIS

In insects, developmental changes of gene expression are most spectacular in the texture, sculpturing and color of the integument. For biochemical analysis, however, the fat body has some advantages. Among the diverse functions of the insect fat body are intermediary metabolism and storage of carbohydrates, lipids and nitrogenous compounds, detoxication, and synthesis of most of the hemolymph proteins (Wyatt, 1975, 1980). During ontogeny it switches roles. In holometabolous larvae, for example, it is responsible for metabolism and blood protein synthesis, while during metamorphosis a storage role becomes predominant, and in the adult it exports proteins that include the precursors of yolk. These roles overlap, but the functional reorganization can be great, and, except in the most advanced groups where there is much cell replacement, it reflects reprogramming in the same fat body cells (Kunkel, 1981).

Among the hormones that regulate the synthesis of specific proteins in fat body, JH is of special interest because of its chemical identity as a sesquiterpenoid, unique among animal hormones, and its powerful multiple roles in regulating metamorphosis and reproduction (Riddiford, 1985; Koeppe et al., 1985). In comparison with ecdysone, its mode of action has been relatively neglected. The synthesis and testing of a vast number of compounds as JH mimics for potential use in pest control (Sehnal, 1983) has brought into focus the problem of the relation of activity to structure. The natural JHs must have the correct enantiomeric form for activity. Methoprene, which was designed to confer enhanced stability to a structure closely resembling JH (Henrick and Staal, 1973; Fig. 1), is highly

Molecular Insect Science, Edited by
H. H. Hagedorn *et al.*, Plenum Press, New York, 1990

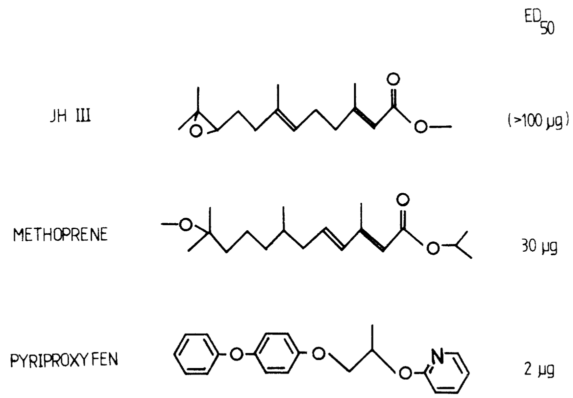


Fig. 1. Juvenile hormone and juvenoids. Structures of JH III, methoprene (Henrick et al., 1973) and pyriproxyfen (= S31183; Hatakoshi et al., 1986), and activities for inducing vitellogenin synthesis in *Locusta migratoria* are shown. Activities were assayed by topical application in acetone to precocene-treated adult female locusts and measurement of either production of vitellogenin by fat body *in vitro*, or of Vg level in hemolymph 3 days after treatment (Dhadialla and Wyatt, 1983; Chinzei and Wyatt, 1985; unpublished data). With JH III, a response was obtained only when a JH esterase inhibitor was injected (Wyatt et al., 1987).

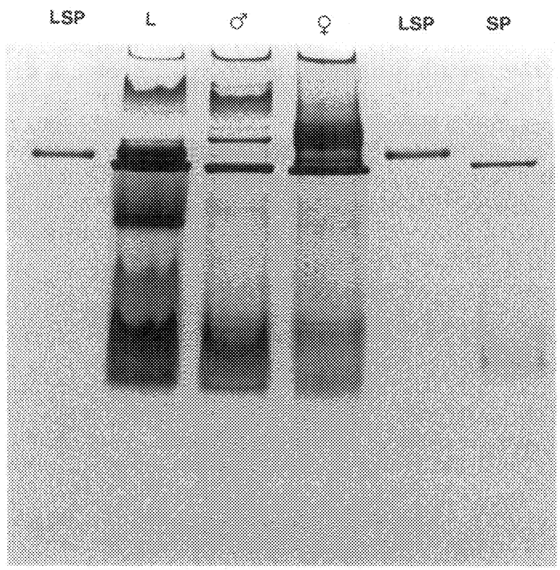


Fig. 2. Locust storage proteins. Hemolymph from stage V day 6 larvae (L), and male and female day 12 adults, together with purified larval storage protein (LSP) and storage protein (SSP), was resolved in non-denaturing 5-25% acrylamide gel electrophoresis with a continuous buffer system. The diffuse, fast-moving bands from hemolymph and purified SSP include SSP dissociation products. Coomassie blue stain. (J. Ancsin and G.R. Wyatt, unpublished).

active. Yet, certain phenoxyether derivatives such as pyriproxyfen (Hatakoshi et al., 1986), which show little apparent resemblance to JH, exhibit still higher levels of activity which are difficult to rationalize. This problem will probably be resolved only when the JH receptor has been characterized and its binding site modelled.

To study JH action on fat body, we have looked chiefly at the control of vitellogenin

(Vg) synthesis in adult female locusts (*Locusta migratoria*; Wyatt, 1988). Initiation of Vg synthesis is correlated with a rise in JH level, is prevented by allatectomy or precocene treatment, and is restored by administration of JH or an active analog. In adults, we have been unable to induce Vg synthesis in males, yet in the fifth larval instar, which does not normally produce Vg, synthesis can be induced by high doses of JH analog in females and also, less strongly, in males, showing that sex-limited programming of the Vg genes is not fully established until after the metamorphic molt. This observation, first made with methoprene (Dhadialla and Wyatt, 1983), has recently been confirmed with pyriproxyfen. In all respects observed, the tissue responses to these two agonists mimic the effects of JH, in accord with the hypothesis that they act by the same primary mechanism.

LOCUST HEMOLYMPH PROTEINS AND EFFECTS OF JUVENILE HORMONE

The protein patterns from denaturing polyacrylamide gel electrophoresis (SDS-PAGE) of female locust hemolymph show, at about day 8 of adult life, when Vg first appears, a simultaneous marked increase in two major bands which migrate at about 80 kDa and 20 kDa (Kanost et al., 1987; Wyatt et al., unpublished). Synthesis of non-Vg protein in the fat body is known to be stimulated by JH (Chen et al., 1979; Gellissen and Wyatt, 1981), and the developmental pattern suggested that this effect may include certain hemolymph proteins. Since the blood proteins of *L. migratoria* have not been adequately described, some purification and characterization was a necessary prelude to testing this idea.

The molecular weight class at 70-80 kDa includes the subunits of the insect storage protein superfamily (Wyatt and Pan, 1978; Levenbook, 1985), as well as apolipophorin II (Chino and Kitazawa, 1981). From locust hemolymph, components of this group of proteins were fractionated by polyethylene glycol precipitation and ion exchange chromatography (J. Ancsin and G. Wyatt, in preparation). For assay of purification, closely-migrating components were resolved by native PAGE under carefully controlled conditions (Fig. 2). Two major hemolymph proteins have been purified and characterized: a larval storage protein (LSP) of 410 kDa with subunits of 75 kDa, and a persistent storage protein (PSP) of 460 kDa, with subunits of 74 and 77 kDa. Our LSP may correspond to the locust larval hemolymph protein of De Kort and Koopmanschap (1987), though its properties are somewhat different from those reported. Another component, previously described (Chino et al., 1983), is a cyanoprotein, which appears as a band migrating very closely behind PSP (Fig. 2, female). These proteins appear to be hexamers, like other insect storage proteins (Levenbook, 1985). PSP dissociates reversibly at pH 9 (as do some dipteran and lepidopteran storage proteins; Roberts and Brock, 1981), and the diffuse, fast-migrating component seen on the native gels represents PSP subunits resulting from dissociation in the electrophoretic buffer. An additional major component, seen in the larval sample as a strong band moving ahead of PSP, remains to be characterized.

Locust LSP, like most insect storage proteins, is larval-specific. Its hemolymph titer rises after day 3 of the fifth instar when the JH titer falls (Baehr et al., 1979), and initial results show that this rise is prevented by treating the locusts with JH analog. Thus, LSP appears to be JH-repressible. This recalls the JH repression of certain storage proteins in lepidopterous larvae (Tojo et al., 1981; Jones, 1988). A JH-repressed protein has also recently been reported in adult cockroach fat body (Bradfield et al., 1989).

PSP is a major protein in adult as well as larval hemolymph and has been shown by amino acid incorporation to be actively synthesized in mature adults of both sexes (Wyatt et al., 1990). Although larval storage proteins persist in the hemolymph of early adults of some insect species, including cockroaches (Lawler and Kunkel, 1974), this may be the first report of synthesis and accumulation of a protein of this class in an adult insect.

After adult locusts were treated with precocene to eliminate endogenous JH, we could examine the effects of a JH mimic on hemolymph titers of several proteins assayed with specific antibodies (Fig. 3). Starting one day after administration of methoprene, Vg appeared and rapidly rose to a high level, as previously reported (Chen et al., 1979; Chinzei and Wyatt, 1985). PSP, which was already present in the JH-deprived insects, increased significantly, nearly doubling its level by day 4. It is therefore produced constitutively but elevated by JH action.

Fractionation of the low molecular weight group of hemolymph proteins also revealed unexpected diversity. Locust apolipophorin III, of 17 kDa, has been described (Chino and

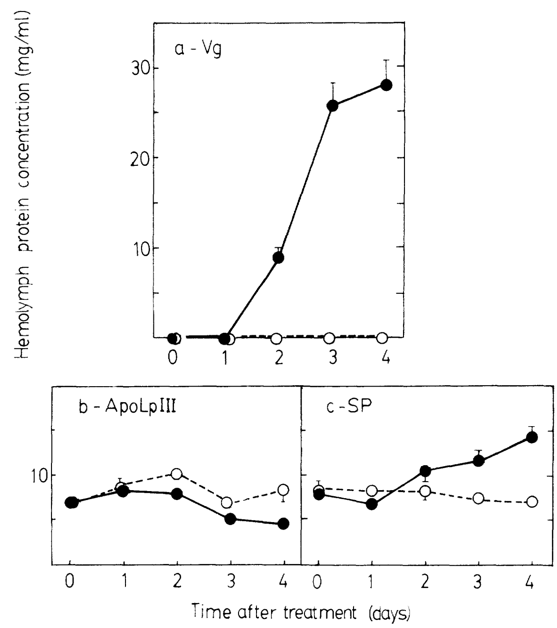


Fig. 3. Effects of JH analog on selected proteins in locust hemolymph. Precocene-treated adult female locusts were treated with methoprene and hemolymph was sampled successively from the same individuals for 4 days. Levels of vitellogenin (Vg), storage protein (SSP) and apolipophorin III (apoLpIII) were determined by rocket immunmoelectrophoresis with specific antisera. -- ● --, methoprene-treated (4 locusts were given 400 µg topically in acetone and 4 were injected with 150 µg in mineral oil); --O--, acetone- and oil-treated controls. Means and S.E.M (n = 8) are shown (Wyatt et al., unpublished).

Table 1. Regulation of synthesis of some locust hemolymph proteins. JH effects were obtained with the JH mimics, methoprene or pyriproxyfen. Repression of LSP was observed in the stage V larvae; other effects were observed in adults. Induction signifies that the protein was not detectable without JH stimulation; elevation signifies that the protein was present without JH but the level or rate of synthesis was elevated by the JH analog.

PROTEIN	STAGE	SEX	JH EFFECTS
LSP	larva	-	repression
PSP	larva, adult	-	elevation
apoLpIII	larva, adult	-	no effect
19K	larva, adult	-	elevation
21K	adult	female	induction
Vg	adult	female	induction

Yazawa, 1986; Kanost et al., 1988a). After JH-deprived locusts were treated with JH analog, it did not rise (Fig. 3), indeed its final level was lower than in controls, possibly because of increase in hemolymph volume. Recently, a distinct 19 kDa protein, initially mistaken for apoLpIII (Konost et al., 1987), was resolved and characterized (Kanost et al., 1988b). By examination of hemolymph PAGE patterns, run under conditions optimized for resolution of native low molecular weight proteins, we have now recognized a 21 kDa monomeric protein which runs in SDS-PAGE together with apoLpIII and the 19 kDa protein (Fig. 4; J.-Z Zhang and G. Wyatt, in preparation). The new 21 kDa protein has been purified. Preliminary data indicate an amino acid composition very similar to that of the 19

kDa protein but with substantially more lys and arg, and an N-terminal sequence closely resembling that of the 19 kDa protein, indicating that the 19 kDa and 21 kDa proteins are related. The regulation of the 21 kDa protein is of special interest: it is female-limited, dependent on JH induction, and synthesized under methoprene stimulation coordinately with Vg (Zhang and Wyatt, unpublished observations). This contrasts with the 19 kDa protein, which is found in both sexes and is produced constitutively but elevated by JH analog (Kanost et al., 1988b).

These locust hemolymph proteins can be grouped with respect to sex limitation and effects of JH (Table 1). Four classes are apparent: (1) female-specific, JH-dependent (Vg and 21K protein); (2) non sex-specific, constitutively synthesized but elevated by JH (PSP and 19K protein); (3) non sex-specific, repressed, in the larval stage, by JH (LSP); (4) non

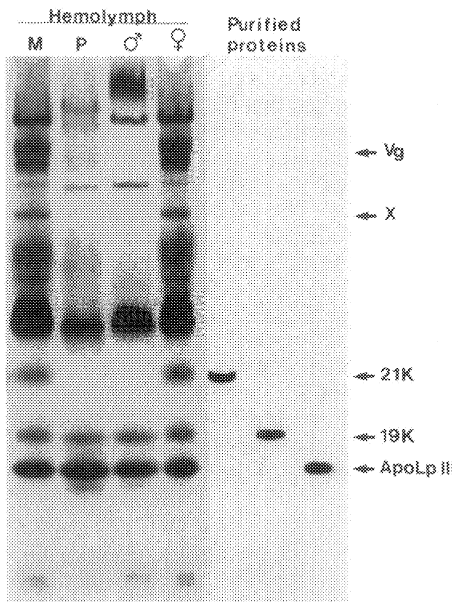


Fig. 4. Low molecular weight proteins of locust hemolymph. Hemolymph from mature male and female locusts, precocene-treated females (P) and precocene-treated females treated with 150 µg methoprene and sampled 4 days later (M), was resolved in non-denaturing 5-15% acrylamide gel electrophoresis with a discontinuous system. Purified 21 kDa protein (21K), 19 kDa protein (19K) and apolipophorin III (apoLpIII) are also shown. X is an uncharacterized female-specific protein. (J.-Z. Zhang and G.R. Wyatt, unpublished).

sex-specific, unaffected by JH (apoLpIII). Comparison of genes for this set of proteins, which are synthesized in the same cells under diverse regulation, will be valuable for identification of control elements.

THE MOLECULAR BIOLOGY OF VITELLOGENIN REGULATION

Our research at the gene level, focussed on Vg, has recently been summarized (Wyatt, 1988). *L. migratoria* has two X-linked Vg genes which exhibit N-terminal sequence homology with one another and with the Vg genes of *Xenopus laevis*, chicken and a nematode. Added to similarity of mRNA size, this strongly suggests that these all belong to a Vg superfamily (Wahli, 1988). In most of the coding region, however, the two locust Vg genes do not cross-hybridize, so that unique probes for their transcripts are available. Assays of transcript accumulation during induction by methoprene show that (1) in the rising phase of induction cellular message level is closely correlated with rate of Vg synthesis,

(2) accumulation of transcripts from the two genes is precisely coordinate, and (3) there is a surprisingly long lag time - about 24 hours in primary induction, reduced to 12 hours in the secondary response - before Vg transcripts are first detectable (Dhadialla et al., 1987). Methoprene penetrates the locust cuticle rapidly, attaining a steady-state level in fat body within 30 minutes (unpublished data), and the delay in Vg transcription is likely to reflect a need for production of an essential gene product. Preliminary experiments with cycloheximide, administered in a dose from which the locusts can recover, indicate that the appearance of Vg mRNA is further delayed by a time interval similar to the duration of inhibition of protein synthesis.

Since the coordinately expressed Vg genes must have equivalent regulatory elements, their upstream flanking sequences were compared, and within 500 bp from the transcription start site, 11 blocks of sequence similarity between the two genes, separated by regions of divergent sequence, were identified (Locke et al., 1987). The close resemblance of two of these to two 12-nucleotide sequence elements found in similar positions upstream from a JH-regulated cockroach oothecin gene has been pointed out by Pau et al. (1987; see also Wyatt, 1988), and it has been suggested that the near-identity of sequences associated with genes for unrelated proteins in distantly related insects may reflect their common regulation by JH. Recently, it has been recognized that the DNA response elements for several steroid and other hormones are remarkably similar to one another, and can be represented as blocks of 12-15 nucleotides that are more or less palindromic with a central spacer (Beato, 1989). In Figure 5, sequences found associated with JH target genes are compared with some known hormone response elements. The consensus estradiol response element (ERE) is a perfect palindrome with a 3-nucleotide spacer. The ecdysteroid consensus (EcRE) has 7 nucleotides identical with this but has lost some of the dyad symmetry. The *D. melanogaster* hsp27 EcRE includes the consensus EcRE and has 8 consecutive nucleotides in its 5'-arm identical with the locust VgA7 sequence. The set of sequences from locust and cockroach JH-regulated genes show marked similarity, especially in the 5'-arms. In the VgA7, VgB7 and Oo1 sequences, symmetry is maximized by including four nucleotides in the central spacer. Three nucleotides are identical in the 5'-arms of all 7 of the insect gene sequences shown. These relationships strongly suggest that the Vg and oothecin sequences shown may be JH response elements. Sequences from additional JH-regulated genes, when available, will provide for further testing of these correlations.

```
ERE          AGGTCA nnn TGACCT
EcRE         AGGGTT nnn TGCACT
hsp27    GACAAGGGTT CAA TGCACTTGTC
VgA7        AAGGGTT CCGT AACTG
VgB7        AAGAGTT CTAT AACAA
Oo1          AGGGTT CCGT AA
VgA11       AATGGTT TTT ATGTCT
VgB11       AATCGAT TTG ATGTTT
Oo2          ATGGAT TTG AGG
```

Fig. 5. DNA sequences that may be JH response elements, compared with known steroid hormone response elements. ERE, consensus estradiol response element; EcRE, consensus ecdysteroid response element (Beato, 1989); hsp27, *Drosophila melanogaster* heat-shock protein ecdysteroid response element (Riddihough and Pelham, 1987); VgA7, VgB7, VgA11, VgB11, sequences upstream from locust vitellogenin genes (Locke et al., 1987); Oo1, Oo2, sequences upstream from cockroach oothecin genes (Pau et al., 1987). Underlined nucleotides show dyad symmetry. In VgA7, VgB7 and Oo1, symmetry is maximized by assuming 4 nucleotides in the central spacer.

STEPS TOWARD A GENE EXPRESSION ASSAY SYSTEM

To obtain functional evidence on JH regulatory elements, an assay system for locust gene transcription is clearly needed. Truncated locust Vg genes inserted into a *P*-element vector and injected into *D. melanogaster* embryos were integrated into the fly genome but not expressed (Wyatt et al., 1986). Transformation of a *D. melanogaster* cell line with a construct of locust Vg upstream DNA and bacterial CAT reporter gene led to weak expression but no enhancement by methoprene (Schreiber, 1987). We are now examining expression from plasmids after injection into early locust embryos. The locust egg is 6-7

mm long and can easily be injected with a glass needle during the first two hours after oviposition. Cleavage is relatively slow in the locust, so that this represents the 2-4 nuclei stage of the syncitial early embryo. For initial characterization of the system, we have used phspCAT (DiNocera and Dawid, 1983), with the strong *D. melanogaster* hsp70 promoter linked to the CAT reporter gene. A moderate level of expression is obtained, from day 3 to day 9 of the 12-day period before hatching, in a proportion of the injected embryos (S. Mathi, unpublished data). We hope that this may be developed into a useful gene expression assay. Other approaches are also being considered.

THE JH RECEPTOR AND DNA-BINDING PROTEINS

The search for the JH receptor has produced many reports on JH-binding proteins from insect cells and tissues (e.g. Chang et al., 1980; Roberts and Wyatt, 1983; Engelmann et al., 1987), but it is difficult to distinguish true receptors, which mediate the primary action of the hormone, from carriers and other more or less specific binding proteins. Recent work with photoaffinity labelling in nuclei from *M. sexta* epidermis is very promising, although differences in the binding of JH itself and methoprene are puzzling (Osir and Riddiford, 1988). Recently, we have demonstrated specific, high-affinity binding of methoprene to locust fat body nuclei (G. Edwards and G. Wyatt, unpublished).

The recognition that the genes for nuclear hormone receptors, including those for steroids, thyroid hormones and other ligands, comprise a homologous superfamily has evoked the suggestion (Evans, 1988) or the assumption (Beato, 1989) that the insect JH receptor belongs to this group. Cloning with the use of a probe from the conserved region of a known receptor gene may now be a productive route to characterization of the JH receptor. By this approach, clones which may code for JH receptors have recently been isolated from *M. sexta* (Riddiford, this volume) and *L. migratoria* (G.M. Tyshenko and G. Wyatt, unpublished).

Specific binding of gene-associated DNA elements by proteins, that include hormone receptors and other transcription factors, can be detected by retardation of electrophoretic mobility of the labelled DNA fragment. By the use of such gel-shift assays, we have demonstrated specifically competable binding of proteins extracted from locust fat body nuclei or whole tissue to several fragments in the region -20 to -440 from the transcription start site of the vitellogenin genes (R. Braun and G. Wyatt, unpublished). Proteins that may exhibit the tissue-, stage- and sex-specificity expected of Vg gene transcription factors are being sought.

HOW DOES JH REGULATE GENE EXPRESSION IN LOCUST FAT BODY?

In locust fat body, the synthesis of several abundant, characterized proteins is affected by JH in different ways, and this tissue represents a good model for analysis of JH action. Two JH-dependent vitellogenin genes are now available, and cloning by standard procedures should soon provide additional genes that are JH-dependent, constitutive but JH-stimulated, JH-repressed, and JH-independent.

For the two Vg genes, the appearance and accumulation of Vg mRNA, closely correlated with the rising synthesis of Vg, is consistent with the regulation of transcription by mechanisms similar to those that are becoming increasingly well understood for steroid and thyroid hormones. These involve direct binding of hormone-receptor complex to DNA response elements upstream from the target genes (Beato, 1989). Potential JHREs have been identified but require confirmation by a functional assay. In the case of the locust Vg genes, however, the prolonged lag time with a requirement for protein synthesis before appearance of Vg transcripts needs to be explained. It is possible that the Vg genes are activated only by the product of another gene which is the immediate target of JH receptor. We propose, however, that the Vg genes are direct JH targets (just as avian and amphibian Vg genes are direct estrogen targets; Klein-Hitpass et al., 1989), but that a synergistically acting product of another gene is also essential. This could be JH receptor or another transcription factor (Schule et al., 1988). The presence of receptor is, of course, required for the initial action of the hormone, and one possibility is that one type of receptor induces production of a second type which in turn recognizes the Vg genes. Characterization of receptors and transcription factors is clearly a high priority. We are now at the point where

continued molecular analysis should soon elucidate the mechanisms by which JH regulates the expression of specific genes.

ACKNOWLEDGMENTS.

It is a pleasure to thank the members of my research group, named herein, for their dedicated efforts, and the Natural Sciences and Engineering Research Council of Canada, the U.S. National Institutes of Health and the Sumitomo Chemical Company Limited, for grant support.

REFERENCES

Baehr, J.-C., Porcheron, P., Papillon, M., and Dray, F. 1979. Haemolymph levels of juvenile hormone, ecdysteroids and protein during the last two larval instars of *Locusta migratoria*. J. Insect Physiol. 25: 415-421.

Baker, B.S. 1989. Sex in flies: the splice of life. Nature 340: 521-524.

Bradfield, J.Y., Berlin, R.L., and Keeley, L.L. 1989. Contrasting modulations of gene expression by a juvenile hormone analog. Insect Biochem. (in press).

Beato, M. 1989. Gene regulation by steroid hormones. Cell 56: 335-344.

Chang, E.S., Coudron, T.A., Bruce, M.J., Sage, B.A., O'Connor, J.D., and Law, J.H. 1980. Juvenile hormone-binding protein from the cytosol of *Drosophila* Kc cells. Proc. Natl. Acad. Sci. USA 77: 4657-4661.

Chen, T.T., Couble, P., Abu-Hakima, R., and Wyatt, G.R. 1979. Juvenile hormone-controlled vitellogenin synthesis in *Locusta migratoria* fat body. Hormonal induction *in vivo*. Dev. Biol. 69: 59-72.

Chino, H., and Kitazawa, K. 1981. Diacylglycerol-carrying lipoprotein of hemolymph of the locust and some insects. J. Lipid Res. 22: 1042-1052.

Chino, H., and Yazawa, M. 1986. Apolipophorin III in locusts: purification and characterization. J. Lipid Res. 27: 377-385.

Chino, H., Abe, Y., and Takahashi, K. 1983. Purification and characterization of a biliverdin-binding cyanoprotein from the locust hemolymph. Biochim. Biophys. Acta 748: 109-115.

Chinzei, Y., and Wyatt, G.R. 1985. Vitellogenin titre in hemolymph of *Locusta migratoria* in normal adults, after ovariectomy and in response to methoprene. J. Insect Physiol. 31: 441-445.

DeKort, C.A.D., and Koopmanschap, A.B. 1987. Isolation and characterization of a larval hemolymph protein in *Locusta migratoria*. Arch. Insect Biochem. Physiol. 4: 191-203.

Dhadialla, T.S., and Wyatt, G.R. 1983. Juvenile hormone-dependent vitellogenin synthesis in *Locusta migratoria* fat body: inducibility related to sex and stage. Dev. Biol. 96: 436-444.

Dhadialla, T.S., Cook, K.E., and Wyatt, G.R. 1987. Vitellogenin mRNA in locust fat body: coordinate induction of two genes by a juvenile hormone analog. Dev. Biol. 123: 108-114.

DiNocera, P., and Dawid, I., 1983, Transient expression of genes introduced into cultured cells of *Drosophila*. Proc. Natl. Acad. Sci. USA. 80: 7095-7098.

Engelmann, F., Mala, M., and Tobe, S.S. 1987. Cytosolic and nuclear receptors for juvenile hormone in fat bodies of *Leucophaea maderae*. Insect Biochem. 17: 1045-1052.

Evans, R.M. 1988. The steroid and thyroid hormone receptor superfamily. Science 240: 889-895.

Gellissen, G., and Wyatt, G.R. 1981 Production of lipophorin in the fat body of Locusta migratoria: comparison with vitellogenin. Can. J. Biochem. 59: 648-654.

Hatakoshi, M., Agui, N., and Nakayama, I. 1986. 2-[1-methyl-2-(4-phenoxyphenoxy) ethoxy] pyridine as a new insect juvenile hormone analogue: induction of supernumerary larvae in *Spodoptera litura*. Appl. Ent. Zool. 21: 351-353.

Henrick, C.A., Staal, G.B., and Siddall, J.B. 1973. Alkyl 3,7,11-trimethyl-2,4-dodecadienoates, a new class of potent insect growth regulators with juvenile hormone activity. Agric. Food Chem. 21: 354-359.

Jones, G., Hiremath, S.T., Hellmann, G.M., and Rhoads, R.E. 1988. Juvenile hormone regulation of mRNA levels for a highly abundant hemolymph protein in larval *Trichoplusia ni*. J. Biol. Chem. 263: 1089-1092.

Kanost, M.R., McDonald, H.L., Bradfield, J.Y., Locke, J., and Wyatt, G.R. 1987. Cloning and expression of the gene for apolipophorin III from *Locusta migratoria*. pp. 275-283 in: "Molecular Entomology." Law, J., ed. UCLA Symp. Mol. Cell. Biol., N.S. Vol. 49. Alan R. Liss, New York.

Kanost, M.R., Boguski, M.S., Freeman, M., Gordon, J.I., Wyatt, G.R., and Wells, M.A. 1988a. Primary structure of apolipophorin III from the migratory locust. *Locusta* migratoria. J. Biol. Chem. 263: 10568-10573.

Kanost, M.R., Bradfield, J.Y., Cook, K.E., Locke, J., Wells, M.A., and Wyatt, G.R. 1988b. Gene structure, cDNA sequence, and developmental regulation of a low molecular weight hemolymph protein from *Locusta migratoria*. Arch. Insect Biochem. Physiol. 8: 203-217.

Klein-Hitpass, L., Tsai, S.Y., Greene, G.L., Clark, J.H., Tsai, M.-J., and O'Malley, B.W. 1989. Specific binding of estrogen receptor to the estrogen response element. Mol. Cell. Biol. 9: 43-49.

Koeppe, J.K., Fuchs, M., Chen, T.T., Hunt, L.-M., Kovalick, G.E. and Briers, T. 1985. The role of juvenile hormone in reproduction. pp. 165-203 in: "Comprehensive Insect Physiology, Biochemistry and Pharmacology." Vol. 8. Kerkut, G.A. and Gilbert, L.I., eds. Pergamon Press, Oxford.

Kunkel, J.G. 1981 A minimal model of metamophosis: fat body competence to respond to juvenile hormone. pp. 107-129 in: "Current Topics in Insect Endocrinology and Nutrition." Bhaskaran, G., Friedman, S. and Rodrigues, J.G., eds. Plenum Press, New York,.

Kunkel, J.G., and Lawler, D.M. 1974. Larval-specific serum protein in the Order Dictyoptera - I. Immunologic characterization in larval *Blattella germanica* and cross-reaction throughout the Order. Comp. Biochem. Physiol. 47B: 697-710.

Levenbook, L. 1985. Insect storage proteins. pp. 307-346 in: "Comprehensive Insect Physiology, Biochemistry and Pharmacology." Vol. 10. Kerkut, G.A. and Gilbert, L.I., eds. Pergamon Press, Oxford.

Locke, J., White, B.N., and Wyatt, G.R. 1987. Cloning and 5' end nucleotide sequences of two juvenile hormone-inducible vitellogenin genes of the African migratory locust. DNA (New York). 6: 331-342.

Osir, E.O., and Riddiford, L.M. 1988. Nuclear binding sites for juvenile hormone and its analogs in the epidermis of the tobacco hornworm. J. Biol. Chem. 263: 13812-13818.

Pau, R.N., Birnstingl, S., Edwards-Jones, K., Gillen, C.U., and Matsakis, E. 1987. The structure and organization of genes coding for juvenile hormone-regulated 16-kDa oothecins in the cockroach, *Periplaneta americana*. pp. 265-277 in: "Molecular Biology of Invertebrate Development." Vol 66. O'Connor, J.D., ed. UCLA Symp. Mol. Cell. Biol. N.S. Alan R. Liss, New York.

Riddiford, L.M. 1985. Hormone action at the cellular level. pp. 37-84 in: "Comprehensive Insect Physiology, Biochemistry and Pharmacology." Vol. 8. Kerkut, G.A. and Gilbert, L.I., eds. Pergamon Press, Oxford.

Riddihough, G., and Pelham, H.R.B. 1987. An ecdysone response element in the *Drosophila* hsp27 promoter. EMBO J. 6: 3729-3734.

Roberts, D.B., and Brock, H.W. 1981. The major serum proteins of dipteran larvae. Experientia 31: 103-110.

Roberts, P.E., and Wyatt, G.R. 1983. Juvenile hormone binding by components of fat body cytosol from vitellogenic locusts. Mol. Cell. Endocrinol. 31: 53-69.

Schreiber, M. 1987. "Vitellogenin Gene Expression in Cultured *Drosophila* cells." M.Sc. thesis. Queen's University, Kingston, Ontario.

Schule, R., Muller, M., Kaltschmidt, C., and Renkawitz, R. 1988 Many transcription factors interact synergistically with steroid receptors. Science 242: 1418-1420.

Sehnal, F. 1983. Juvenile hormone analogues, pp. 657-672 in: "Endocrinology of Insects." Downer, R.G.H. and Laufer, H., eds. Alan R. Liss, New York.

Tojo, S., Kiguchi, K., and Kimura, S. 1981. Hormonal control of storage protein synthesis and uptake by the fat body in the silkworm, *Bombyx mori*. J. Insect Physiol. 27: 491-497.

Wahli, W. 1988. Evolution and expression of vitellogenin genes. Trends Biochem. Sci. 4: 227-232.

Wyatt, G.R. 1975. Regulation of protein and carbohydrate metabolism in insect fat body. Verh. Dtsch. Zool. Ges. 1974: 209-226.
Wyatt, G.R. 1980. The fat body as a protein factory. pp. 201-225 in "Insect Biology in the Future." Locke, M., ed. Academic Press, New York.
Wyatt, G.R. 1988. Vitellogenin synthesis and the analysis of juvenile hormone action in locust fat body. Can. J. Zool. 66: 2600-2610.
Wyatt, G.R., and Pan, M.L. 1978. Insect plasma proteins. Annu. Rev. Biochem. 47: 779-817.
Wyatt, G.R., Kanost, M.R., Locke, J., and Walker, V.K. 1986. Juvenile hormone-regulated locust vitellogenin genes: lack of expression after transfer into *Drosophila*. Arch. Insect Biochem. Physiol. Suppl. 1: 35-46.
Wyatt, G.R., Cook, K.E., Firko, H., and Dhadialla, T.S. 1987. Juvenile hormone action on locust fat body. Insect Biochem. 17: 1071-1074.

VERTEBRATE-TYPE NEUROPEPTIDES AND STEROIDS IN *Locusta migratoria*: IDENTIFICATION AND METABOLISM

Arnold De Loof, J. Vanden Broeck, L. Swevers and L. Schoofs

Zoological Institute
Naamsestraat 59
B-3000 Leuven, Belgium

INTRODUCTION

The search for "vertebrate-type" steroid hormones in insects was initiated in our lab after Huybrechts and De Loof (1977, 1982) succeeded in inducing vitellogenin synthesis in male flies (*Sarcophaga bullata, Calliphora erythrocephala, Musca domestica, Phormia regina*) by injecting or feeding 20-hydroxyecdysone. Flies apparently use this steroid as the equivalent of estrogens in vertebrates, which use this steroid for control of vitellogenin synthesis by the liver. When the search for a male-specific ecdysteroid did not yield any positive result, the question was raised whether insects might perhaps have some typical "vertebrate-type" steroids. There is now hard evidence that at least 10 different such steroids are indeed present in insects. Not clear at all is whether these steroids derive either from food or from biosynthesis. In a parallel line of research, the possible presence of molecules resembling the peptide hormones that control steroid biosynthesis in vertebrates (e.g. LH, FSH, ACTH) was investigated. Immunocytochemical data suggest that they are indeed present. None of these has as yet been isolated, however, but as an indirect result, a lot of other neuropeptides have been fully characterized.

Non-ecdysteroid steroids in insects: open questions

The presence in a few insect species of about a dozen of these steroids has been unequivocally demonstrated by gas chromatography-mass spectrometry. In our lab, the following steroids have been identified : pregnenolone, progesterone, 17 α-hydroxyprogesterone, 17 α-hydroxy-20 β-dihydroprogesterone, dehydroepiandrosterone, androst-5-ene-3 β,17 β-diol, androstenedione, testosterone, 5 α-dihydrotestosterone, 5 α-androstane-3 β,17 β-diol, 5 β-androstane-3 α,17 β-diol, 11 β-hydroxytestosterone, 11-ketotestosterone and estradiol (De Clerck et al., 1984, 1988; Novak and Lambert, 1989).

By means of radio-immunoassay (RIA), immunoreactivity has been found in all species examined. With this method, however, there is always the possibility of cross-reaction with other related but different substances. Clear and reproducible time-dependent changes of pregnenolone-like substances have been observed during metamorphosis of *S. bullata*. Methoprene, a synthetic juvenile hormone analogue, has pronounced effects on these steroid concentrations during metamorphosis (Novak, 1989). In *L. migratoria,* concentrations of six different steroids have been measured using RIA in tissues and hemolymph during larval and adult development (Novak et al., 1987; Paesen, 1989). No clear pattern of changes in steroid concentrations according to tissue and developmental stage could be observed. Hitherto, no correlations have been found between fluctuations of steroid titers and various physiological processes. A major question is whether these steroids are biosynthesized or originate from the food. Some vertebrate-type steroids do occur in plants, but only in low concentrations. It is possible that some of them pass the alimentary tract and circulate in the

body. They probably are rapidly metabolized, as injected radiolabeled pre-cursors are rather quickly converted to other steroids and/ or conjugates. In *S. bullata,* however, the levels of immunoreactive steroids remain quite high during the 12 days of metamorphosis, although they are metabolized rapidly. Thus, either there is a continuous supply of new steroids not derived from the food, or the RIA measures other molecules. In *L. migratoria*, the major experimental animal in our lab, the possible presence of steroidogenic enzymes in the gonads and other organs has been investigated. The enzymes found are "metabolic enzymes" such as 17 β-hydroxysteroid dehydrogenase (HSD), 20 α-HSD and 20 β-HSD while important biosynthetic enzymes such as 17 α-hydroxylase and aromatase are not present (Fig. 1). If there is biosynthesis, it does not follow the pathways commonly used by vertebrates.

To find out which steroids might have a function, the presence of receptors in the different tissues has been investigated. Only for pregnenolone and progesterone (both C21 steroids) could a binding protein be found in the male copulatory organ (Kd = 4.5 10^{-8} M) (Paesen et al., 1988) and the spermatheca of femal (Kd = 4.9 10^{-8} M) (Paesen and De Loof, 1988), respectively.

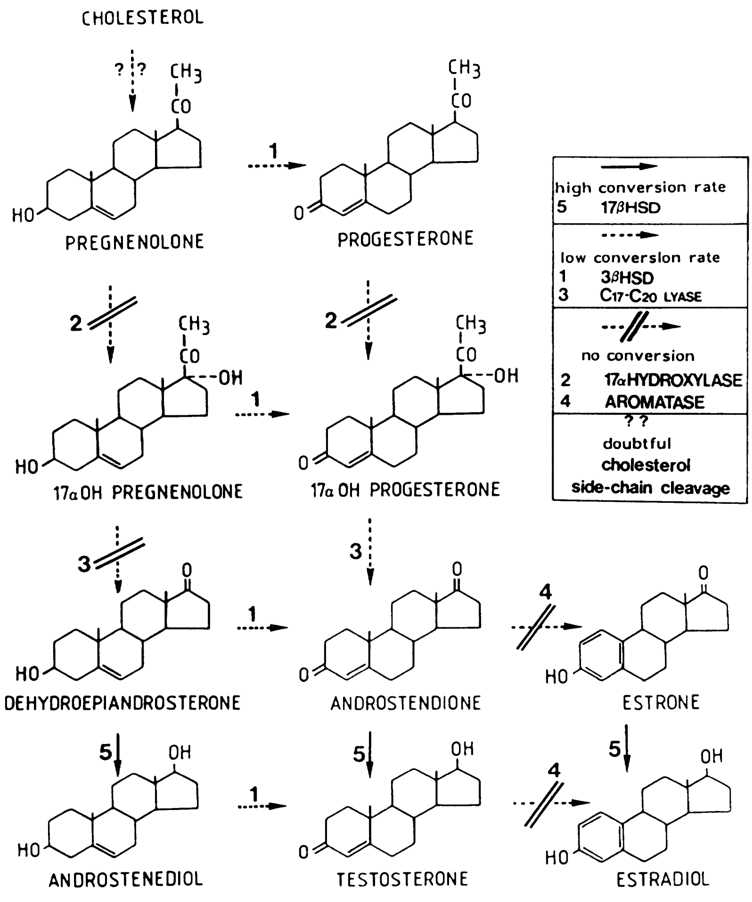


Fig. 1. Steroidogenic enzymes in the gonads of the migratory locust. The absence of androgen and estrogen synthesis from pregnenolone in the gonads of *Locusta migratoria*. Two important enzymatic conversions could not be demonstrated: 17α-hydroxylase (2) and aromatase (4). Two other important biosynthetic enzymes: 3β-hydroxysteroid dehydrogenase (HSD) (1) and C17-C20 lyase (3), demonstrated low activities. Gonads of the locust, however, were capable of converting C17-keto androgens or estrogens rapidly to their corresponding 17β-hydroxy metabolites [17β-HSD activity (5)]. Finally, the conversion of cholesterol to pregnenolone has not been demonstrated.

We still face the possibility that the insect species we examined have yet another set of steroids that do not occur in vertebrates, but which might be of more physiological importance. In our metabolic studies, dozens of steroid metabolites were found which displayed chromatographic behavior different from that of all common vertebrate steroid metabolites. Rather recently, transcription factors belonging to the Zinc-finger family have been isolated from *D. melanogaster* (Segraves, 1989) and a *H. cecropia* cell line (Kafatos, in press). These transcription factors display great homology (to more than 50 % in the Zinc-finger region) with the mammalian steroid/thyroid/retinoic acid receptors. They do not bind ecdysteroids and it is still not clear what their ligand(s) might be. Steroids continue to be possible ligand candidates, but it can not be excluded that, in insects, non-steroidal molecules are the physiological equivalent of the sex steroids in vertebrates. In our opinion, a physiological role for the typical vertebrate steroids (progesterone, estradiol, corticosteroids) in insects seems unlikely, at least in the species we have investigated. Despite all of the research that has been performed to date, we can only speculate on the chemical nature of the ligand(s) of these putative receptors.

THE SEARCH FOR NEW NEUROPEPTIDES IN *Locusta migratoria*

The chromatographic approach

After a Melanophore Stimulating Hormone (MSH)-related substance with chromatographic behavior similar to that of amphibian MSH was found (Schoofs et al., 1988), 10,000 brains of *L. migratoria* were collected and kept frozen at -70°C until extraction. When it turned out that the methanol-acetic acid extracts displayed biological activity in the *Leucophaea maderae* hindgut bioassay (ie. in a heterologous assay), the decision was taken to try to isolate the active substances. In addition to proctolin, 44 active fractions were purified. Of these, 32 stimulate the motility of the *L. maderae* hindgut while 12 have an inhibitory effect. For comparison, 12 stimulatory and 1 inhibitory substances have been isolated from *L. maderae*. All active fractions are peptides, as shown by amino acid analysis. Ten stimulating peptides have been sequenced: 5 belong to the leucopyrokinin family, 1 is related to gastrin/CCK, and 4 belong to the tachykinin family (Table 1). So far only 1 inhibitory fraction has been sequenced. It is a 9 amino acid-peptide, which does not display any similarity with the presently known invertebrate and vertebrate peptides.

The recombinant-DNA approach

L. migratoria brains were microdissected. RNA was extracted from these brains and selected for poly-A rich sequences on oligo-dT cellulose columns. The eluted RNA mixture was tested by an *in vitro* translation experiment in a rabbit reticulocyte-lysate system. Starting from these poly-A enriched RNAs, double-stranded cDNA was prepared according

Table 1. Primary structures of 6 myotropic peptides of *Locusta migratoria*

Lom-Pk	pGlu-Asp-Ser
	-Gly-Asp-Glu-Trp-Pro-Gln-Gln-Pro-Phe-Val-Pro-Arg-Leu-NH_2
Lom-MT	Gly-Ala-Val-Pro-Ala-Ala-Gln-Phe-Ser-Pro-Arg-Leu-NH_2
Lom-MTII	Glu-Gly-Asp-Phe-Thr-Pro-Arg-Leu-NH_2
Tachykinin-like:	
Lom-TK I	Gly-Pro-Ser-Gly-Phe-Tyr-Gly-Val-Arg-NH_2
Lom-TK II	Ala-Pro-Leu-Ser-Gly-Phe-Tyr-Gly-Val-Arg-NH_2
Gastrin-CCK-like:	
Lom-SK	pGlu-Leu-Ala-Ser-Asp-Asp-Tyr-Gly-His-Met-Arg-Phe-NH_2
	SO_3H

to Gubler and Hoffman (1983) and inserted into the phage vector λgt 11 (Young and Davis, 1983; Huynh et al., 1985). The library was screened with antisera directed against several vertebrate and a few known invertebrate peptide hormones. The antisera chosen have previously given positive results when used in immunocytochemical studies on locust brain tissue sections (see e.g. De Loof, 1987). Immunoreactive cDNA-clones were selected by means of antisera raised against bovine somatotropin (growth hormone, bGH), α-melanotropin (α-MSH), the hypertrehalosemic hormone (HTF) of the stick insect *Carausius morosus,* and the oviposition hormone (CDCH) of the snail *Lymnea stagnalis.* No reaction was obtained with antisera directed against human insulin, somatostatin (S.R.I.F., somatotropin release inhibiting factor), chicken somatotropin, or luteinizing hormone (LH). Immunopositive clones were further purified and subcultured. The cDNA inserts were analyzed by restriction-enzyme digestion, hybridization and sequence determination.

So far, no spectacular nucleic acid or derived amino-acid sequence homologies have been observed between the immunopositive locust cDNA clones and the original antigens or corresponding genes. Usually only limited similarities between small sequence fragments containing 3 or 4 amino acids were found, as is shown in Table 2. The best resemblance was observed between a 13-amino-acid sequence fragment of a CDCH-immunopositive clone and part of the precursor polypeptide of this oviposition hormone (CDCH) of *Lymnea stagnalis.* About 62% of local homology was found between these partial sequences (Table 3). Although it is not entirely clear whether the selected cDNA clone corresponds to a sequence that is evolutionary related to the CDCH precursor gene of *Lymnea stagnalis*, the discovery of this high degree of local homology is a very interesting result.

The general conclusion of our study is that the likelihood of finding pronounced homology between the selected sequence and the (foreign) ligand against which the antiserum was prepared is rather low,. and cross-reactions may occur. Especially when rare sequences are to be discovered, this phenomenon may be a big problem. Such immunological cross-reactions, however, most probably have also taken place in some of the above mentioned immunocytochemical studies. Several immunohistochemical data can clearly prove this.

More specific detection might be expected when using well characterized antisera and monoclonal antibodies. These antibodies, however, might have difficulty recognizing the cDNA expression products, which are unprocessed chimeric proteins. The structural difference between a biologically active peptide and the corresponding λgt 11 expression product may therefore result in altered immunological binding properties. Moreover, the antisera employed were directed not against endogenous *L. migratoria* peptide hormones, but

Table 2 . Amino-acid sequence fragment similarities observed by comparing immunopositive locust cDNA clones with the original antigens against which the antisera were directed.

a) HTF (hypertrehalosemic factor of *Carausius morosus*):

pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp-Gly-Thr-NH2

b) locust brain cDNA clones immunoreactive to HTF:

-Leu-Ser-Phe-Thr-
-Thr-Leu-Phe-Thr-

c) Amino-acid sequence fragments derived from HTF immunoreactive locust cDNA sequences:

bGH (bovine somatotropin) sequence fragments:	versus	locust brain cDNA clones immunoreactive to bGH
-Leu-Ser-Gly-Leu-Phe-		-Leu-Thr-Leu-Leu-Phe-
-Phe-Lys-Glu-Phe-		-Phe-Lys-Val-Phe-
-Ser-Arg-Val-		-Ser-Arg-Val-
-Arg-Phe-Gly-Glu-Ala-Ser-		-His-Phe-Gly-Glu-Glu-Ser-
-Ser-Ile-Gln-Asn-Thr-Gln-		-Ser-Asp-Glu-Asp-Ser-Gln-

Table 3. Comparison made between amino-acid and nucleic acid sequence fragments of the *Lymnea stagnalis* oviposition hormone (CDCH) precursor and of the corresponding immunopositive *CDCH Locusta migratoria* clone.

CDCH-immunopositive clone (*Locusta migratoria) :*

```
-Ala-Arg-Ser-Pro-Arg-Ala-Ser-Arg-Ser-Ala-Pro-Ser-Ser-
 GCA AGA AGT CCA AGA GCA TCG AGA TCC GCA CCA AGT TCC     Locusta

 TCC CGA AGT CGC AGA TCG GCC GGC TCC GCA CCG AGC TCG     Lymnea
-Ser-Arg-Ser-Arg-Arg-Ser-Ala-Gly-Ser-Ala-Pro-Ser-Ser-
```

against peptides of a foreign origin. In spite of these possible immunological recognition problems, this screening method can lead to the characterization of yet unknown insect brain factors and their genes. Monoclonal antibodies (or well defined polyclonal antisera), made against the locust material itself, might yield good results, as has been clearly shown by Lagueux et al. (in press), who found *L. migratoria* 'insulin' cDNA in this way.

In a second approach, oligonucleotide probes were synthesized "against" two newly identified *L. migratoria* myotropins, namely Lom-SK (*L. migratoria* sulfakinin) and Lom-PK (L. *migratoria* pyrokinin). These peptides were selected because of the lower degree of genetic code degeneracy corresponding with their amino-acid sequences. The probes were labeled by kinase treatment in the presence of γ-^{32}P-ATP and used to screen the cDNA library by means of hybridization. No matching sequences were obtained under the hybridization conditions employed for these oligonucleotide mixtures. The hybridization conditions used for these probe mixtures were probably not optimal, and it is possible that the required sequences are so rare that a very large number of primary phage clones will have to be tested.

In a third (related) approach, recently introduced, the PCR (polymerase chain reaction) technique was used to clone the cDNAs corresponding to these two peptides. In this very reliable method, which allows the rapid and specific amplification of extremely rare DNA sequences, oligonucleotide primers are used to initiate DNA synthesis reactions. These reactions are performed by means of a temperature-stable enzyme : Taq polymerase. The enzyme remains active during the many consecutive temperature cycles the reaction mixture has to undergo. During the temperature cycles optimal reaction conditions are successively created for DNA denaturation, annealing, and synthesis. The cyclic change of the reaction conditions causes the amplification effect.

Single-stranded cDNA was prepared according to Frohman et al. (1988). The length of the cDNA strands was checked by alkaline agarose electrophoresis. The oligonucleotide primer sequences were derived from the amino acid sequences of the above-mentioned locust peptide hormones. In order to obtain an efficient and specific amplification, the reaction conditions had to be optimized. A variety of conditions were tested : the quantities of cDNA and primers, Mg^{2+} concentration in the buffers, temperature cycling conditions and enzyme quantity were varied. The amplified DNA products were then further analyzed by polyacrylamide electrophoresis and visualized by ethidium bromide fluorescence. Once the conditions were optimal, distinct DNA bands could be observed. This method can be used specifically to amplify certain rare transcripts.

DISCUSSION

The work done so far on non-ecdysteroid steroids did not yield conclusive results as to their physiological significance. The origin of these steroids remains unclear. The discovery of transcription factors belonging to the Zinc family (Kafatos, in press; Segraves, 1989), which display a high degree of homology to the typical steroid receptors in vertebrates, but which do not bind ecdysone, suggests the existence of a new class of ligands, which might perhaps be steroids, different from the typical vertebrate-type steroids. Our incubation experiments with radiolabeled steroid precursors have shown that *L. migra-*

toria can make a large number of as yet unidentified steroid metabolites, which have as yet not been described in vertebrates. It is tempting to speculate on a physiological role for some of these unknown steroids.

The high number of myotropic new peptides, originating from the brain of *L. migratoria*, clearly shows that the insect brain probably contains hundreds of different regulatory peptides. This does not mean that all of the identified peptides have a hormonal function. Some of them may exert their actions in the nervous system itself. We have reached the level where we can safely say that there are indeed structural relationships between some regulatory peptides of vertebrates and insects, a matter of dispute for several years. The comparative approach opens a lot of new possibilities for discovering new peptides. The result will be that the textbook schemes for endocrine control of several physiological processes in insects are most probably far too simple. In general, physiological processes seem to be controlled by a balance of hormonal factors rather than in a single one. This requires models of hormone action other than the ones presently available. Such a model, based on the self-electrophoresis principle, has already been put forward a few years ago. This model points to the numerous possibilities, offered by the plasma membrane and perhaps also the nuclear envelope, for endocrine control of gene expression and certain physiological processes (De Loof, 1986 a,b).

ACKNOWLEDGMENTS

This research was supported by the I.W.O.N.L., N.F.W.O., Queen Elizabeth of Belgium Foundation, the Research Foundation of the Catholic University, Leuven and NATO.

REFERENCES

De Clerck, D., Diederik, H., and De Loof, A. 1984. Identification by capillary gas chromatography-mass spectrometry of eleven non-ecdysteroid steroids in the haemolymph of larvae of *Sarcophaga bullata*. Insect Biochem. 14: 199-208.

De Clerck, D., Diederik, H., Paesen, G., and De Loof, A. 1988. Identification and quantification of C21 and C19 steroids in the haemolymph of *Leptinotarsa decemlineata*, a phytophagous insect. Insect Biochem. 18: 93-99.

De Loof, A. 1986a. The electrical dimension of cells: The cell as a miniature electrophoresis chamber. Int. Rev. Cytol. 104: 251-352.

De Loof, A. 1986b. Tissue specificity of hormone action: A new theory. Insect Biochem. 16: 169-173.

De Loof, A. 1987. The impact of the discovery of vertebrate-type steroids and peptide hormone-like substances in insects. Entomol. Exp. Appl. 45: 105-113.

Frohman, M.A., Dush, M.K., and Martin, G.R. 1988. Rapid production of full-length cDNAs from rare transcripts: Amplification using a single gene-specific oligonucleotide primer. Proc. Natl. Acad. Sci. USA 85: 8998-9002.

Gubler, U., and Hoffman, B.J. 1983. A simple and very efficient method for generating cDNA libraries. Gene 25: 263-269.

Huybrechts, R., and De Loof, A. 1977. Induction of vitellogenin synthesis in male *Sarcophaga bullata* by ecdysterone. J. Insect Physiol. 23: 1359-1362.

Huybrechts, R., and De Loof, A. 1982. Similarities in vitellogenin and control of vitellogenin synthesis within the genera *Sarcophaga, Calliphora, Phormia* and *Lucilia*. Comp. Biochem. Physiol. 72B: 339-344"

Huynh, T.V., Young, R.A., and Davis, R.W. 1985. "DNA Cloning, A Practical Approach." Vol. I. Glover, D.M., ed. IRL Press Oxford, Washington.

Kafatos, F.C. Gene regulatory mechanisms and their evolution : formation of the insect eggshell. in: "Advances in Invertebrate Reproduction 5." Elsevier, Amsterdam. in press.

Lagueux, M., Lwoff, L., Goltzen, F., Meister, M., Hietter, H., Zachary, D., Holder, F., Charlet, M., and Hoffmann, J. Studies on the isolation and characterization of neuropeptides involved in the control of reproduction in adult females of *Locusta migratoria*. Meeting Abstract, 5th International Congress of Invertebrate Reproduction, Nagoya, Japan. in press.

Novak, F. 1989. Identification and physiology of non-ecdysteroid steroids in a few arthropod species. Ph.D. Thesis. Catholic University, Leuven, Belgium.

Novak, F., De Clerck, D., Paesen, G., Swevers, L., and De Loof, A. 1987. Radioimmunological quantification of C21, C19 and C18 steroids in haemolymph of the insect *Locusta migratoria*. Int. J. Invert. Reprod. Devel. 11: 255-264.

Novak, F.J.S., and Lambert, J.G.D. 1989. Pregnenolone, testosterone and estradiol in the migratory locust, *Locusta migratoria*: A gas chromatographic-mass spectrometric study. Gen. Comp. Endocrinol. 76: 73-82.

Paesen, G. 1989. Search for the significance of C18, C19 and C21 steroids in *Locusta migratoria* by means of radioimmunoassays, radioreceptorassays and *in vitro* incubation experiments. Ph.D. Thesis. Catholic University, Leuven, Belgium.

Paesen, G., and De Loof, A. 1988. The presence of a progesterone binding protein in spermathecae of the migratory locust, *Locusta migratoria migratorioides*. Invert. Reprod. Devel. 14: 267-277.

Paesen, G., Novak, F., Swevers, L., De Clerck, D., and De Loof, A. 1988. The presence of a pregnenolone-binding factor in the copulatory organ of the migratory locust, *Locusta migratoria migratorioides*. Gen. Comp. Endocrin. 72: 402-407.

Schoofs, L., Jégou, S., Tonon, M.-C., Huybrechts, R., Vaudry, H., and De Loof, A. 1988. Biological, chromatographical and radioimmunological evidence for a melanotropin-like peptide in the central nervous system of *Locusta migratoria*. Gen. Comp. Endocrinol. 71: 36-44.

Segraves, W.A. 1989. E75, an expression-inducible regulator of developmental gene expression. Meeting Abstract, IXth Ecdysone Workshop, Paris, France.

Young, R.A., and Davis, R.W. 1983. Efficient isolation of genes using antibody probes. Proc. Natl. Acad. Sci. USA. 80: 1194-1198.

NEUROPEPTIDES OF MOSQUITOES

Arden O. Lea and Mark R. Brown

Department of Entomology
University of Georgia
Athens GA 30602, USA

INTRODUCTION

The adult mosquito, despite the limitations of its size, is a valuable experimental animal for the study of insect reproduction and such related processes as feeding behavior, digestion, water balance, anabolism, and the endocrine regulation of these processes. Mosquitoes could hardly escape our attention, since they are vectors of most of the transmissible pathogens that are lethal to several millions of humans each year. For many years, physiologists have been attracted to mosquitoes by the obvious association between their blood-feeding habits and oocyte maturation. Yet, the most salient question of how the ingestion of vertebrate blood signal the onset of oogenesis, remains only partially answered after nearly a half century of investigation.

The earliest attempts to discover the link between a blood meal and oogenesis were the head ligation and decapitation experiments of Detinova (1945), Clements (1956), and Gillett (1956). The results implicated the head in the regulation of mosquito oogenesis but did not indicate whether oogenesis was blocked because a nervous signal did not reach the brain or because a humoral factor was not released from the head. Later, Lea (1972) showed that, when a blood-fed decapitated female was joined to an intact non-blood-fed female, both of the parabiotic partners matured oocytes. This was the clearest demonstration that a humoral factor, rather than nerves responding to the midgut stretched with blood, was the transducing signal from the diet.

In 1952, Ellen Thomsen published a landmark paper demonstrating that, in *Calliphora erythrocephala*, the medial neurosecretory cells (MNC) in the pars intercerebralis play a key role in oogenesis. Here, the extirpation of the MNC of newly eclosed flies prevented vitellogenesis during subsequent protein-feeding. After learning Thomsen's surgical procedures for *C. erythrocephala*, Lea (1967) applied them to mosquitoes and confirmed the importance of the MNC in mosquito oogenesis. Implantion of the MNC into the abdomen of a female in which vitellogenesis was blocked by MNC-ablation, restored the ability of the female to complete oocyte maturation. However, the specific target of the postulated "egg development neurosecretory hormone" (EDNH) remained unknown.

Then in 1975, Hagedorn and his colleagues reported that mosquito ovaries secrete ecdysteroids after a blood meal and later demonstrated that a factor from the brain stimulates ecdysteroid secretion by the ovaries (Hagedorn et al., 1979). Hagedorn refers to this factor as EDNH. While we have confirmed that a factor from the MNC stimulates ovarian secretion of ecdysteroids *in vitro* (Brown and Lea, unpublished), there are many steps in the synthesis of vitellogenin by the fat body and its sequestration by the oocytes (see Raikhel and associates, this conference), and there are probably several factors from the MNC that regulate these steps and other physiological processes. Therefore, this may be an

Molecular Insect Science, Edited by
H. H. Hagedorn *et al.*, Plenum Press, New York, 1990

appropriate time to eliminate the indefinite term EDNH and refer, more precisely, to a specific factor from the MNC that is an "ovarian ecdysteroidogenic hormone" (OEH).

While recent interest in the fat body has centered on its synthetic capacity for yolk proteins, earlier work focused on the storage of energy reserves from the alternate food source, nectar. In the female, but not the male mosquito, a sugar meal is converted into a limited amount of glycogen, with the excess sugar going to triglycerides (Van Handel, 1965). Extirpation of the MNC removed the normal limit on glycogen synthesis, while implantation of 4 pairs of MNC, into an MNC-ablated female, reset the limit for glycogen accumulation (Lea and Van Handel, 1970). Removal of the MNC had the opposite effect on the synthesis of triglycerides after a sugar meal, but we could not demonstrate hormonal involvement in the regulation of triglyceride synthesis, because implanted MNC did not restore the normal condition.

Mosquitoes have also been favored experimental animals for studies of water balance, because of the enormous water-load acquired with a blood meal. Illustrative of this problem are the anecdotal reports that a horse or cow, tethered overnight in an area with a high mosquito population, would sometimes be found in the morning surrounded by a dark ring of blood-engorged mosquitoes. These females had taken so much blood that they would only glide off the animal into the grass. Here, they could only hop away, just beyond the limit of the hooves of the tethered animal. By ingesting two and sometimes three times her own body weight during blood-feeding, the female obviously has a critical water balance problem that requires immediate correction. Her response is a very rapid discharge of urine for the first 30 to 60 min, followed by a slower constant rate of excretion during digestion. Decapitation, immediately after the ingestion of blood, abolishes the rapid phase of urination in *Anopheles freeborni* (Nijhout and Carrow, 1978). These investigators also showed that extracts of heads and the thoracic nervous system stimulated rapid urine formation in isolated Malpighian tubules, suggesting that a factor(s) from the CNS stimulates urine secretion. Thus, the evidence in mosquitoes favors endocrine regulation of diuresis rather than neural control as suggested by Stobbart (1977).

Mosquito larvae have perhaps attracted even more attention than adults for water balance studies, because some fresh water species can tolerate highly brackish water. Maddrell and Phillips (1978) demonstrated that isolated Malpighian tubules of fed larvae of *Aedes taeniorhynchus* secrete fluid at a higher rate than those from unfed larvae. They also showed that the addition of an extract of larval brains to the incubation medium stimulated rapid secretion by isolated tubules. Extracts of the brain were about 4 times more active than extracts of thoracic ganglia, while abdominal ganglia were inactive. Interestingly, they reported that the larval brain, incubated in a potassium enriched solution, known to induce secretion of diuretic factors in *Rhodnius prolixus* and *Glossina morsitans*, released a factor that stimulated fluid secretion in the isolated tubule preparation of *A. taeniorhynchus*. Thus, elimination of excess water in larvae seems to be under the control of a diuretic hormone, probably from neurosecretory cells in the brain.

With the techniques for purification of small quantities of peptides that have become available in the last few years, we can use the accumulated physiological information, and the tantalizing hints of endocrine factors released from the CNS, to attempt their purification and characterization. But before describing peptide isolation, we wish to call attention to the midgut as a potential source of equally important regulatory peptides.

In the early 1980s, we decided, as part of our investigation of the regulation of oogenesis, to examine the midgut for the source of whatever signal indicates to a female that "protein is in the midgut and vitellogenin synthesis should begin." An electron microscopic search of the midgut by Brown et al. (1985) revealed more than 500 peptidergic cells scattered throughout both the thoracic and abdominal parts of the mosquito midgut. Some of these cells were similar to a type first reported for mosquitoes by Hecker (1971 a,b). With immunocytochemical methods and antisera to a number of vertebrate peptide hormones and to Phe-Met-Arg-Phe-amide (FMRF-amide), the molluscan cardioaccelerator, Brown et al. (1986) showed that about 200 of these endocrine-like cells in the abdominal region of the midgut were immunoreactive to FMRF-amide antiserum.

While searching for reactive cells in the midgut, we discovered many FMRF-amide immunoreactive cells in the CNS. In fact, the brain contains cells reactive not only to FMRF- amide but also to antisera against adipokinetic hormone (Brown and Lea, 1988), leu-enkephalin, dynorphin, and urotensin (Brown et al., this conference).

PEPTIDE PURIFICATION

We decided first to extract the FMRFamide-like material from mosquito heads, as there would be less tissue to extract and heads would be more easily collected than abdomens. This approach necessitated rearing several hundred thousand mosquitoes per week, saving only females, and collecting and stockpiling their heads at -80° C. Using a FMRF-amide antiserum in a radioimmunoassay to detect immunoreactive fractions from chromatography, Dr. Shogo Matsumoto, working in our laboratory, developed a purification protocol that yielded three related peptides from heads of *Aedes aegypti*. These were purified to homogeneity and sequenced (Matsumoto et al., 1989a):

Aea-HP-I	pGlu-Arg-Pro-HyPro-Ser-Leu-Lys-Thr-Arg-Phe-amide
Aea-HP-II	Thr-Arg-Phe-amide
Aea-HP-III	pGlu-Arg-Pro-Pro-Ser-Leu-Lys-Thr-Arg-Phe-amide

These three peptides share the -Arg-Phe-amide (RF-amide) carboxyl-terminal sequence with FMRF-amide and with the myotropic leucosulfakinins (Nachman et al., 1986) and the inhibitory leucomyosuppressin (Holman et al., 1986) isolated from cockroaches. *Aea*-HP-I is also similar to the bradykinins and vespakinin-M with their -Arg-Pro-(Hy)Pro- sequences.

Aedes-Head Peptide-I

To confirm the identity of *Aea*-HP-I, we had it synthesized and showed that its chromatographic properties were identical to those of the native peptide. After conjugating the peptide to thyroglobulin, we then made an antiserum in rabbits, which we used in an immunocytochemical investigation of the distribution of reactive cells in adults, larvae, and pupae.

In the midgut of 3-day-old females, there are several hundred *Aea*-HP-I immunoreactive cells scattered throughout the abdominal portion of the midgut. In addition, over a hundred cells in the CNS, including some of the medial neurosecretory cells, are also immunoreactive. By comparative immunocytochemistry, we found that *Aea*-HP-I had the same distribution in the CNS and midgut as FMRF-amide, and by testing the antisera to FMRF-amide and *Aea*-HP-I on adjacent sections, we observed that both bound to the same cells. Therefore, we now suspect that, because the two peptides have the same carboxyl-terminal sequence of -Arg-Phe-amide, the immunoreactive peptide in the CNS, and possibly the midgut cells, is *Aea*-HP-I or a homolog and not FMRF-amide. However, until we can compare the sequence of *Aea*-HP-I with whatever midgut peptide is immunoreactive to its antiserum, we cannot conclude that the same peptide is secreted from both the CNS and midgut cells.

In the last instar larva, a few cells, reactive to *Aea*-HP-I antiserum, are present in head, thoracic, and abdominal ganglia with the majority of the immunoreactive cells in the posterior region of the midgut. In contrast, immunoreactive cells are not observed in the differentiating midgut of newly eclosed pupae, but there are a few strongly immunoreactive cells in the adult-like ganglia in the head.

The antiserum to *Aea*-HP-I (1/100,000 final dilution) was also used in a sensitive radioimmunoassay (5-50 Fmol/ml) to measure quantities of peptide in various adult tissues, including the hemolymph. We discovered that a female contains 5-10 pmol/female at three days post-eclosion. This amount of peptide is almost equally divided between the head and midgut.

In time course studies, the peptide titer in hemolymph increases from 0.8-0.9 pmol to 2.4-3.2 pmol/μl during the first 40 h after a blood meal and, thereafter, drops to the initial titer, independently of oviposition.

In trying to determine the function(s) of *Aea*-HP-I, we have used this peptide in a dozen or more biological assays, but the only effect has been in muscle contraction assays, where it suppresses contractions of isolated hindguts from crickets (Matsumoto, unpublished) and *Heliothis zea* (Crim et al., 1989). However, with the variation in the titer of *Aea*-HP-I immunoreactive material circulating after a blood meal, we are not convinced that myosupression is the only or primary function of this neuropeptide, and we continue to look for other hormonal or modulatory effects.

Diuretic and Antidiuretic Factors from Adult A. aegypti

Experiments by Wheelock et al., (1988) document the fact that the head is the source of factors that affect diuresis in blood-fed *A. aegypti*. The isolation and characterization of diuretic factors from heads of this species are now a joint undertaking between the laboratories of Klaus Beyenbach, Timothy Hayes, and Henry Hagedorn. Three active fractions, isolated by HPLC from crude extracts of heads, are heat stable but inactivated by treatment with pronase (Petzel et al., 1986). Isolated Malpighian tubules respond to fractions II and III by increased fluid secretion (Petzel et al., 1987). Fraction III hyperpolarizes the transepithelial voltage of an isolated Malpighian tubule and stimulates a nearly l0-fold increase in intracellular cyclic adenosine monophosphate (cAMP), thereby mimicing the effect of exogenous cAMP on NaCl secretion by the tubules. Because this peptide selectively stimulates NaCl secretion and increase intracellular levels of cAMP, it is referred to as a mosquito natriuretic factor (Petzel et al., 1985).

Other peptides, tested on Malpighian tubules of *A. aegypti*, have shown either diuretic or antidiuretic activity. Recently, Hayes et al.. (1989) reported that the eight myotropic leukokinins from cockroaches, stimulate Malpighian tubule activity. The *Aea*-HP-I has no diuretic activity in mosquitoes (Beyenbach and Brown, unpublished). Therefore, the structural characterization of the mosquito diuretic peptides will be of considerable interest, because of a possible sequence homology between the leukokinin family of peptides and mosquito diuretic factors.

Mosquitoes, like other small terrestrial animals, are in constant danger of desiccation, and as a consequence, water balance must be maintained by an interplay of antidiuretic factors, with the diuretic peptides just described. In fact, we have encountered several chromatographic fractions from mosquito head extracts that contain powerful inhibitors of diuresis *in vivo*, and we are currently purifying them for characterization.

Oostatic Hormone

Meola and Lea (1972) described experiments that suggested the ovaries of females retaining eggs secreted a factor that blocked further oogenesis, if the female refeeds on blood. Presumably this factor was similar to the oostatic hormone of Adams et al., (1968) and the antigonadotropic hormone of Huebner and Davey (1973). Borovsky (1985) extracted an "oostatic" factor, from the ovaries of blood-fed mosquitoes, that partially arrested egg development and reduced the number of eggs laid after injection of females subsequently fed blood. Later, Borovsky (1988) showed that this factor was a peptide, which blocked synthesis of trypsin in the midgut. He and Carlson (this conference) now report that the factor is a decapeptide which they have synthesized and shown that, *in vivo*, it also inhibits secretion of trypsin-like enzymes in the midgut. Thus, as the factor increases in titer after a blood meal, reaching a maximum at 48 hr, it appears to function by regulating the termination of digestion rather than serving expressly as an oostatic hormone. This is more compatible with the fact that females, which refeed on blood while retaining eggs, do digest the second blood meal completely, albeit somewhat more slowly (Briegel and Lea, unpublished).

Ovarian Ecdysteroidogenic Hormones in A. aegypti

To return to the ovarian ecdysteroidogenic hormone(s) of the adult mosquito, there have been several attempts to purify and characterize this important hormone. This continuing struggle, which began with Hanaoka and Hagedorn (1980), is summarized in Table 1.

One impediment to progress has likely been insufficient starting material for purification. Preliminary estimates by Matsumoto in our laboratory indicate that several hundred thousand heads would be required to purify and characterize OEH. For the purification, Matsumoto used both conventional chromatography and HPLC. Initially, our *in vivo* assay was the autogenous *Aedes atropalpus* system of Fuchs (Table 1.), but later we changed to *A. aegypti*, decapitated within a few minutes of blood-feeding. With this new assay, we identified several active fractions that were negative in the *A. atropalpus* assay. We also used the Hanaoka-Hagedorn *in vitro* assay to verify that active fractions from the *in vivo* assay would stimulate ecdysteroid secretion *in vitro*, as measured in the radioimmunoassay described by Warren et al. (1984).

Table 1. Summary of efforts to purify ovarian ecdysteroidogenic hormones.

EXTRACT OF	ASSAY	PURIFICATION METHODOLOGY	MOL. WT. (DALTONS)
9,600 heads (M & F)	a	conventional chromatography (1)	6,500
? heads	b	dialysis (2)	3,500-12,000
15,000 heads (M & F)	a	reversed phase HPLC (3)	
? heads (F)	a & c	reversed phase HPLC (4)	
95,000 whole bodies (M & F)	b & d	conventional chromatograpy & electrophoresis (5)	18,750
? heads (M & F)	a & b	gel filtration (6)	11,000 & 24,000
800,000 heads (F)	a, b, e	conventional chromatography and reversed phase HPLC (7)	6,500-13,000

a) Ovaries of non-blood-fed *Aedes aegypti* incubated 6 h with extract; ecdysteroids measured with RIA.
b) Autogenous *Aedes atropalpus* decapitated within 1-2 h of eclosion and injected with extract; yolk in oocytes judged visually 72 h after eclosion.
c) *Aedes aegypti* decapitated 2 h after a blood meal and injected with extract; 52 h after meal, vitellin in oocytes measured by rocket immunoprecipitation.
d) Abdomen ligated after a blood meal and treated with 12.5 pg methoprene followed by injection of extract; yolk in oocytes measured visually 45 h later.
e) *Aedes aegypti* fed blood and decapitated within 30 m and injected with extract; yolk in oocytes measured visually 48 h after feeding.

(1) Hanaoka & Hagedorn 1980; (2) Fuchs et al. 1980; (3) Masler et al. 1983; (4) Wheelock & Hagedorn 1985; (5) Borovsky & Thomas 1985; (6) Whisenton et al. 1987;
(7) Matsumoto et al. 1989b.

From an extract of more than 880,000 heads, Matsumoto et al., (1989b) separated three groups of fractions that stimulated vitellogenesis and ecdysteroid secretion. The molecular weights of the active peptides from one of the three groups were between 6.5 and 13 kDa, and these peptides were purified to homogeneity for structural studies. Analysis of the amino acid composition of one of these peptides revealed that it was a basic peptide, confirming its observed chromatographic properties, with at least 92 amino acid residues.

Several attempts to sequence these active peptides from the amino-terminal have been unsuccessful, and we now assume that this terminal is blocked. Other attempts to sequence the peptides after digestion with different enzymes were also unsuccessful, until we were able recently to obtain a partial sequence from a peptide fragment. This fragment has 40% residue identity to the intron DNA sequence 9 bases downstream from the STOP codon after the first residue of amino acid 26 in the B-chain of human insulin-like growth factor I. Additional sequence analyses are needed to confirm the relatedness of OEH to bombyxin (Nagasawa et al., 1986), melanization and reddish coloration hormone (Matsumoto et al. 1985), and other insulin-like peptides.

Is adult OEH the same peptide as "prothoracicotropic hormone" (PTTH) of larvae? This question is of interest to all who have worked with OEH. Kelly et al. (1986) have already tested a partially purified extract of *A. aegypti* heads for its ability to stimulate secretion of ecdysone by larval and pupal prothoracic glands of *Manduca sexta in vitro*, and

"big" and "small" PTTH, partially purified from *M. sexta* pupal brains, for their activity in adult *A. aegypti*. They concluded that there was no cross-stimulation between these factors in the two insects.

Prothoracicotopic Hormone

Although there has been no experimental evidence for PTTH in mosquito larvae, the hormone is assumed to exist and initiate ecdysteroid secretion and molting. With our mass-rearing facility and a protocol for purifying OEH in hand, we have an unusual opportunity to identify mosquito PTTH and compare the larval and adult ecdysteroidogenic neurohormones.

We are using two assays to test chromatographic fractions from extracts of larval heads of *A. aegypti* for PTTH. In one, molting, suppressed in last instar larvae by dietary manipulation, can be stimulated when larvae are injected with a crude extract of larval heads or 20-hydroxyecdysone (a positive control). In the other assay, ecdysteroid secretion by cultured prothoracic glands is being measured by radioimmunoassay.

CONCLUSION

Our long-term goal, of course, is to compare the primary structure of OEH and PTTH of *A. aegypti*. However, considering the number of fractions with OEH activity (Matsumato et al., 1989b), it appears likely that the adult has several species of OEH, perhaps in varying quantities, potencies, and even with subtle differences in functional targets. Nevertheless, we are confident that with extraction of several million heads and alternative methods of peptide fragmentation, as well as by employing techniques to identify the OEH gene, we will be able to determine the structure of these important mosquito peptides.

Adult mosquitoes clearly continue to be valuable animals with which to study the regulation of diuresis, carbohydrate metabolism, and reproductive processes. Therefore, because of the investment in the time and effort to mass rear and extract mosquitoes, we are utilizing the inactive material from the OEH purification to identify other neuroendocrine factors, as well as providing material to collaborators. However, if new peptides are purified with immunoassays, the difficulty will be to find their biological function. Immunological recognition does not guarantee either structural or functional similarity to the vertebrate peptide antigen. On the other hand, these peptides will give us specific tools with which to search for biological functions and may provide clues to the evolution of some of these regulatory molecules.

While the CNS will clearly be the source of new neuropeptides, we predict that the midgut will also provide many regulatory peptides. Furthermore, we should not overlook the practical implications of the midgut being the interface between the diet and internal tissues and, therefore, a likely source of signals controlling feeding, resting behavior, and key metabolic processes (Brown and Lea, 1990).

ACKNOWLEDGMENTS

We especially thank Mr. D. M. Fendley for his dedicated microsurgical assistance. This work was supported by a grant (AI-17297) from the National Institute of Health.

REFERENCES

Adams, T. S., Hintz, A. M. and Pomonis, J. G. 1968. Oostatic hormone production in house flies, *Musca domestica*, with developing ovaries. J. Insect Physiol. 14: 983-993.

Borovsky, D. 1988. Oostatic hormone inhibits biosynthesis of midgut proteolytic enzymes and egg development in mosquitoes. Arch. Insect Biochem. Physiol. 7: 187-210.

Borovsky, D. 1985. Isolation and characterization of highly purified mosquito oostatic hormone. Arch. Insect Biochem. Physiol. 2: 333-349.

Borovsky, D. and Thomas, B. R.. 1985. Purification and partial characterization of mosquito egg development neurosecretory hormone: evidence for gonadotropic and

steroidogenic effects. Arch. Insect Biochem. Physiol. 2: 265-281

Brown, M. R. and Lea, A. O. 1988. FMRFamide- and adipokinetic hormone-like immunoreactivity in the nervous system of the mosquito, *Aedes aegypti*. J. Comp. Neuro. 270: 606-614.

Brown, M. R. and Lea, A. O. 1990. Neuroendocrine and midgut endocrine systems in the adult mosquito. in: "Advances in Disease Vector Research." K. F. Harris, ed. Springer-Verlag, New York (in press).

Brown, M. R., Raikhel, A. S. and Lea, A. O. 1985. Ultrastructure of midgut endocrine cells in the adult mosquito *Aedes aegypti*. Tissue Cell. 17: 709-721.

Brown, M. R., Crim, J. W. and Lea, A. O. 1986. FMRFamide- and pancreatic polypeptide-like immunoreactivity of endocrine cells in the midgut of a mosquito. Tissue Cell. 18 (3): 419-428.

Clements, A. N. 1956. Hormonal control of ovary development in mosquitoes. J. Exp. Biol. 33: 211-223.

Crim, J. W., Jenkins, A. C, Brown, M. R., Herzog, G. A. and Lea, A. O.. 1989. FMRF-amide in the corn earworm (*Heliosthis zea*): Immunoreactivity in the midgut and cerebral nervous system. in: "Proc. International Conf. Insect Neurochem. and Neurophysiol." (In press).

Detinova, T. S. 1945. On the influence of glands of internal secretion upon the ripening of the gonads and the imaginal diapause in *Anopheles maculipennis*. Zoologsky Zharnal.. 24: 297-298.

Fuchs, M. S., Sundland, B. R. and Kang, S.-H.. 1980. *In vivo* induction of ovarian development in *Aedes atropalpus* by a head extract from *Aedes aegypti*. Int. J. Invert. Reprod. 2: 121-129.

Gillett, J. D. 1956. Initiation and promotion of ovarian development in the mosquito *Aedes (Stegomyia) aegypti*. Ann. Trop. Med. Parasitol. 50: 375-380.

Hagedorn, H. H., O'Connor, J. D., Fuchs, M. S., Sage, G., Schlaeger, D. A. and Bohm, M. K. 1975. The ovary as a source of α-ecdysone in an adult mosquito. Proc. Natl. Acad. Sci. USA 72: 3255-3259.

Hagedorn, H. H., Shapiro, J. P. and Hanaoka, K. 1979. Ovarian ecdysone secretion is controlled by a brain hormone in an adult mosquito. Nature 282: 92-94.

Hanaoka, K., and Hagedorn, H. H. 1980. Brain hormone control of ecdysone secretion by the ovary in a mosquito. pp. 467-480 in: "Progress in Ecdysone Research". J. A. Hoffman, ed. Amsterdam: Elsevier/North Holland.

Hayes, T. K., Pannabecker, T. L., Hinckley, D. J., Holman, G. M., Nachman, R. J., Petzel, D. H. and Beyenbach, K. W. 1989. Leucokinins, a new family of ion transport stimulators and inhibitors in insect malpighian tubules. Life Sci. 44: 1259-1266.

Hecker, H., Freyvogel, T. A., Briegel, H. and Steiger, R. 1971a. Ultrastructural differentiation of the midgut epithelium in female *Aedes aegypti* imagines. Acta Trop. 28: 80-104.

Hecker, H., Freyvogel, T. A., Briegel, H. and Steiger, R. 1971b. The ultrastructure of midgut epithelium in *Aedes aegypti* males. Acta Trop. 28: 275-290.

Holman, G. M., Cook, B. J. and Nachman, R. J. 1986. Isolation, primary structure and synthesis of Leucomyosuppressin, an insect neuropeptide that inhibits spontaneous contraction of the cockroach hindgut. Comp. Biochem. Physiol. 85: 329-333.

Huebner, E. and Davey, K. G. 1973. An antigonadotropin from the ovaries of the insect *Rhodnius prolixus*. Can. J. Zoo. 51(2): 113-119.

Kelly, T. J., Whisenton, L. R., Katahira, E. J., Fuchs, M. S., Borkovec, A. B. and Bollenbacher,.W. E. 1986. Inter-species cross-reactivity of the prothoracicotropic hormone of *Manduca sexta* and egg-development neurosecretory hormone of *Aedes aegypti*. J. Insect Physiol. 32: 757-762.

Lea, A. O. 1967. The medial neurosecretory cells and egg maturation in mosquitoes. J. Insect Physiol. 13: 419-429

Lea, A. O. 1972. Regulation of egg maturation in the mosquito by the neurosecretory system: the role of the corpus cardiacum. Gen. Comp. Endocr. Suppl. 3: 602-608.

Lea, A. O. and Van Handel, E. 1970. Suppression of glycogen synthesis in the mosquito by a hormone from the medial neurosecretory cells. J. Insect Physiol. 16: 319-323.

Maddrell, S. H. P. and Phillips, J. E. 1978. Induction of sulphate transport and hormonal control of fluid secretion by malpighian tubules of larvae of the mosquito *Aedes taeniorhynchus*. J. Exp. Biol. 72: 181-202.

Masler, E. P., Hagedorn, H. H., Petzel, D. H. and Borkovec, A. B. 1983. Partial purifica-

tion of egg development neurosecretory hormone with reverse-phase liquid chromatographic techniques. Life Sci. 33: 1925-1931.
Matsumoto, S., Isogai, A. and Suzuki, A. 1985. N-terminal amino acid sequence of an insect neurohormone, melanization and reddish coloration hormone (MRCH): heterogeneity and sequence homology with insulin-like growth factor II. FEBS Lett. 189: 115-118.
Matsumoto, S., Brown, M. R., Crim, J. W., Vigna, S. R. and . Lea, A. O. 1989a. Isolation and primary structure of neuropeptides from the mosquito, *Aedes Aegypti*, immunoreactive to FMRFamide antiserum. Insect Biochem. 19: 277-283.
Matsumoto, S., Brown, M. R., Suzuki, A. and Lea, A. O. 1989b. Isolation and characterization of ovarian ecdysteroidogenic hormones from the mosquito, *Aedes aegypti*. Insect Biochem. 19: 651-656..
Meola, R. and Lea, A. O. 1972. Humoral inhibition of egg development in mosquitoes. J. Med. Ent. 9: 99-103.
Nachman, R. J., Holman, G. M., Haddon, W. F. and Ling, N. 1986. Leucosulfakinin, a sulfated insect neuropeptide with homology to gastrin and cholecystokinin. Science 234: 71-73.
Nagasawa, H., Kataoka, H., Isogai, A., Tamura, S., Suzuki, A., Mizoguchi, A., Fujiwara, Y., Suzuki, A., Takahashi, S. Y. and Ishizaki, H. 1986. Amino acid sequence of a prothoracicotropic hormone of the silkworm *Bombyx mori*. Proc. Natl. Acad. Sci. USA 83: 5840-5843.
Nijhout, H. F., Carrow, G. M. 1978. Diuresis after a bloodmeal in female *Anopheles freeborni*. J. Insect Physiol. 24: 293-298.
Petzel, D. H., Hagedorn, H. H. and Beyenbach, K. W. 1985. Preliminary isolation of mosquito natriuretic factor. Am. J. Physiol. 18: R379-R386.
Petzel, D. H., Hagedorn, H. H. and Beyenbach, K. W. 1986. Peptide nature of two mosquito natriuretic factors. Am. J. Physiol. 19: R328-R332.
Petzel, D. H., Berg, M. M. and Beyenbach, K. W. 1987. Hormone-controlled cAMP-mediated fluid secretion in yellow-fever mosquito. Am. Physiol. Soc. 20: R701-R711.
Stobbart, R. H. 1977. The control of the diuresis following a blood meal in females of the yellow fever mosquito *Aedes aegypti*. J. Exp. Biol. 69: 53-85.
Thomson, E. 1952. Functional significance of the neurosecretory brain cells and the corpus cardiacum in the female blow-fly *Calliphora erythrocephala*. J. Exp. Biol. 29: 137-172.
Van Handel, E. 1965. The obese mosquito. J. Physiol. 181: 478-486.
Warren, J. T., Smith., W. and Gilbert, L. I. 1984. Simplification of the ecdysteroid radioimmunoassay by the use of protein A from *Staphlococcus aureus*. Experentia 40: 393-394.
Wheelock, G. D. and Hagedorn, H. H. 1985. Egg maturation and ecdysiotropic activity in extracts of mosquito (*Aedes aegypti*) heads. Gen. Comp. Endocrinol 60(2): 196-203.
Wheelock, G. D., Petzel, D. H., Gillett, J. D., Beyenbach, K. W. and Hagedorn, H. H. 1988. Evidence for hormonal control of diuresis after a blood meal in the mosquito *Aedes aegypti*. Arch. Insect Biochem. Physiol. 7: 75-89.
Whisenton, L. R., Kelly, T. J. and Bollenbacher, W. E. 1987. Multiple forms of cerebral peptides with steroidogenic functions in pupal and adult brains of the yellow fever mosquito, *Aedes aegypti*. Mol. Cell. Endocrinol. 50: 3-14.

LOCUST ADIPOKINETIC HORMONES: MOLECULAR BIOLOGY OF BIOSYNTHESIS

Michael O'Shea, Siegfried Hekimi and Marie-Francoise Schulz-Aellen

Laboratory of Cell Biology
University of London, New College
Egham, Surrey, UK.

INTRODUCTION

Adipokinetic hormone is the name given to a biological activity detectable in extracts taken from the locust corpora cardiaca, major neuroendocrine structures in insects (Mayer and Candy, 1969; Beenakkers, 1969). In recent years there has been a great deal of progress at the molecular level concerning these and related hormones in other insects and several primary structures are known. The corpora cardiaca (CC) of the locust have moreover provided us with a model preparation in which to investigate the molecular biology of neuropeptide biosynthesis.

The adipokinetic activity found in the locust CC is produced by two different but related peptide hormones called AKH I (Stone et al., 1976) and AKH II (Siegert et al., 1985). In recent years structurally related hormones have been discovered in the CC of other insects. A selection of insect hormones with the AKH signature is given in Table 1.

While in the locust the adipokinetic hormones cause the liberation of diacylglycerols from the fat body into the circulation and are in general involved in the activation of lipid metabolism (see Orchard, 1987 for review), this is not the case for the similar peptides of other insect species. For example, in the cockroach, *Periplaneta americana* the AKH-like peptides named MI and MII (O'Shea et al, 1984; Witten et al, 1984), are involved in stimulating carbohydrate metabolism (Siegert et al, 1986). In many of the species two forms of an AKH- related peptide are found in the CC. The biological significance of this is not

Table 1. Selected AKH Family Peptides.

GENUS	NAME	SEQUENCE
Schistocerca	AKH I	pGlu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-Thr-NH2
Schistocerca	AKH II-S	pGlu-Leu-Asn-Phe-Ser-Thr-Gly-Trp-NH2
Locusta	AKH II-L	pGlu-Leu-Asn-Phe-Ser-Ala-Gly-Trp-NH2
Periplaneta	M I	pGlu-Val-Asn-Phe-Ser-Pro-Asn-Trp-NH2
Periplaneta	M II	pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp-NH2
*Manduca**	M-AKH	pGlu-Leu-Thr-Phe-Thr-Ser-Ser-Trp-Gly-NH2
*Heliothis**	H-AKH	

*Manduca and Heliothis AKH share the same primary structure (Ziegler et al 1985; Jaffe et al 1986).

Molecular Insect Science, Edited by
H. H. Hagedorn *et al.*, Plenum Press, New York, 1990

clear because only one function can be attributed to the two forms. It seems unlikely that the CC would manufacture two different peptides unless there were distinct and exclusive functions. Biology, however, has unfortunately proven more difficult than chemistry and the gap between our knowledge of the biological activities of the AKH peptides on the one hand and their molecular structures on the other continues to widen. It has widened further recently because molecular cloning and protein sequencing techniques have been applied to the study of AKH biosynthesis (Hekimi et al, 1989a; Schulz-Aellen et al, 1989). This has led to the identification of the AKH precursors and novel peptides of as yet unknown function that are co-synthesized, co-stored and co-released with the AKHs. These are parts of the AKH precursors and named AKH Precursor Related Peptides or APRPs (Hekimi and O'Shea, 1989).

This brief essay will add very little to our understanding of the functional significance of either the adipokinetic hormones or the co-synthesized APRPs. Our main purpose is to illustrate how useful the CC of the locust have been in allowing us to probe the molecular and cellular mechanisms of AKH biosynthesis. Special features (described below) of the locust CC have facilitated this analysis and have provided important new insights into neuropeptide synthesis which might be difficult to gain from any other system. Moreover if insect peptidergic systems offer new potential targets for insect control, the CC will provide the model in which novel compounds which interact with the molecular machinery of peptidergic systems can be tested (O'Shea, 1986; 1985).

SITE OF AKH AND APRP SYNTHESIS IN THE LOCUST CORPORA CARDIACA

The CC are the major organs of the insect neuroendocrine system that store neurohormones and release them into the circulation. As their name implies, they are intimately associated with the heart vessel and are therefore well placed to distribute hormones rapidly throughout the animal. The neurohormones secreted by the CC are synthesized by neurosecretory cells contained both in the brain and within the CC themselves. In the locust the neurosecretory cells intrinsic to the CC are clustered together in the so-called glandular lobes (Fig. 1). Neurosecretory cells of the brain send axons to the CC through two pairs of nerves and these axons arborize in separate lobes called the storage lobes.



Fig. 1. Frontal section of the CC stained with Mayer's hemalumeosin. The glandular lobes (GL) consist of densely packed neurosecretory cells. SL = storage lobe, N = nerve.

The adipokinetic hormones are located in and synthesized by the intrinsic neurosecretory cells of the glandular lobes (Goldsworthy et al, 1972a, b; Hekimi and O'Shea, 1987). These are typical neurosecretory cells appearing to have short axons with cell bodies approximately 50 microns in diameter. Several hundred of them are packed

closely together in the glandular lobes and each is packed with large numbers of electron dense secretory granules (Rademakers and Beenakkers, 1977; Krogh and Normann, 1977) which have been shown by differential centrifugation to contain adipokinetic activity (Stone and Mordue, 1979). Several lines of evidence show that individual neurosecretory cells of the CC make both AKH I and AKH II. This can be seen clearly by immunocytochemical labelling of the glandular lobe using antibodies with high specificity for AKH I and AKH II (Hekimi and O'Shea, 1989b). All cells of the glandular lobe in the adult locust are labelled by both AKH I and AKH II specific antibodies (Fisher-Lougheed et al, unpublished observations). With respect to AKH I and AKH II biosynthesis therefore, the neurosecretory cells of the CC are a homogeneous population of peptide producing cells--an extremely useful and unusual feature as most neural tissues are chemically very heterogeneous at the cellular level. The locust CC can therefore be considered to be a peptide factory, much like the ELH producing bag cells of Aplysia (Scheller et al, 1982; 1983) or the insulin producing β-cells. It is as if the glandular lobes contained a clonal cell line in which the hormone producing genes were specifically and significantly up-regulated. Unlike an artificially constructed cell line, however, the glandular lobes are a tissue in which there is appropriate gene expression and regulation. We have exploited this system to understand the molecular and cell biology of AKH biosynthesis and its regulation.

PHYSIOLOGICAL ACTIONS AND RELEASE

The actions of peptides that share the AKH structural signature are diverse but appear in general to be concerned with increased metabolic activity. For example, some members of the AKH family are involved in stimulating carbohydrate metabolism, others act directly on muscle to favour lipid oxidation and some are cardioacceleratory. In the locust AKH activity has primarily been studied in the adult in which it regulates lipid metabolism during long-distance migratory flight. Flight appears to be an important stimulus for the release of adipokinetic hormones in adult locusts and the releasing factor may be octopamine, an amine produced in the brain by some of the neurosecretory cells which project to the storage lobe of the CC (Orchard and Loughton, 1981a, b). This picture of AKH function in the adult unfortunately does not explain the need for two forms of AKH, nor does it help us understand why the AKH peptides are present in all wingless larval stages.

Concerning the physiological function of the peptides co-synthesized with the AKHs (the APRPs), we as yet have no direct evidence that they are hormones. The APRPs however are co-released with the AKHs (Hekimi and O'Shea, 1989b) so it seems likely that they are hormones, perhaps with an activity related to flight behavior in the adult. An as yet untested hypothesis is that the APRPs are diuretic hormones--an idea suggested by the observation that during flight, when the locust is metabolizing lipid, metabolic water is produced. Locusts, like other insects, have impermeable cuticles so this additional water load, if not lost via respiration, must be removed by excretion. Indeed, it has been observed that during long-term flight locusts do excrete water, a physiological activity presumably provoked by a diuretic hormone. These arguments suggested to us that the APRPs are involved with water balance in some way. This hypothesis has yet to be tested.

THE MOLECULAR BIOLOGY, PROTEIN CHEMISTRY AND CELL BIOLOGY OF AKH BIOSYNTHESIS

We now have a fairly complete picture of how the locust adipokinetic hormones are made (Figure 2). The model is complicated somewhat by the fact that the immediate precursors of AKH I and II are not linear prohormones but dimeric constructs. This surprising (to us) and unprecedented feature of peptide hormone biosynthesis was discovered by characterizing the hormone precursors by direct protein chemistry as well as molecular cloning.

The dimer precursors of AKH I and II are formed from two prohormones called the A-chain (pro-AKH I) and the B-chain (pro-AKH II) which are encoded by two messenger RNAs called respectively mRNA-A and mRNA-B (Hekimi et al, 1989a; Schulz-Aellen et al, 1989). Both mRNAs have been sequenced from clones identified and isolated from cDNA library made from CC mRNA. Clones were identified by their hybridization to oligonucleo-

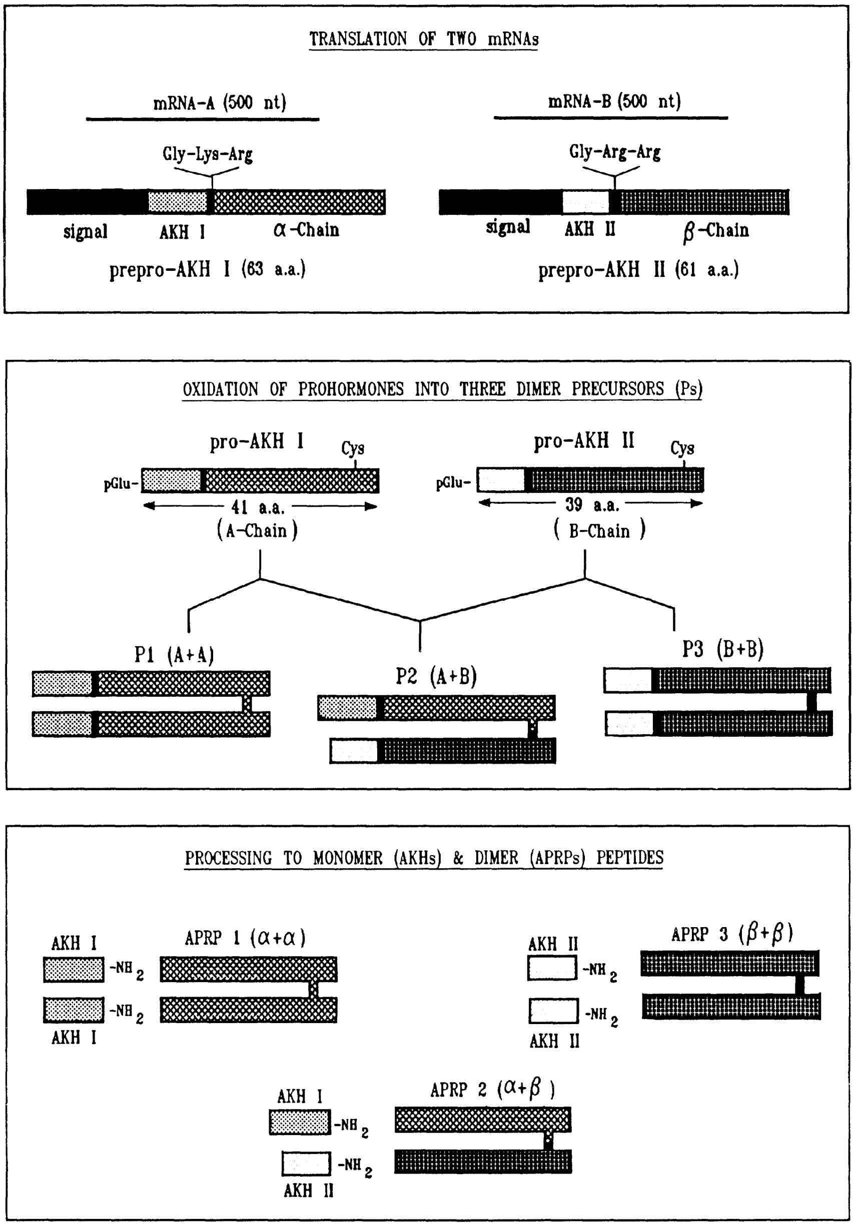


Fig. 2. A model of AKH I, AKH II and APRP biosynthesis from three dimer precursors P1, P2 and P3. nt = nucleotides; aa = amino acids. For explanation see text.

tides based on the amino acid sequences of the AKH and APRP peptides. The sequence of mRNA-A translates into a prepro-AKH I inferred protein consisting of a 22 amino acid signal peptide, followed by the 41 residue A-chain. The A-chain is AKH I, a Gly-Lys-Arg processing site and a 28 residue peptide called the α-chain. This was followed by a stop codon in the cDNA. The protein inferred from the mRNA-B cDNA clone indicated a prepro-AKH II protein consisting of a 22 residue signal peptide (similar to but not the same as the mRNA-A signal), followed by the B-chain (Schulz-Aellen et al, in preparation). The B-chain is AKH II, a processing site, and a 28 residue peptide called the β-chain. The 28 residue β-peptide shares about 70% homology with the α-chain. Amino acid sequences of the A and B-chains were determined by protein sequencing after pulse-chase experiments conducted on *in vitro* cultured CC identified the precursors of the AKHs. Inferred (from cloned cDNA) and determined (from purified precursors) sequences of precursors correspond exactly.

The presence of signal or leader peptide sequences up-stream from the peptide hormone sequences is common in secreted proteins (Sossin et al, 1989). It guides the translated protein into the lumen of the endoplasmic reticulum where processing occurs (Walter and Blobel, 1981). An early post-translational event is thought to be the removal of the signal peptide and in our system this would release A- and B-chains into the endoplasmic reticulum. The A- and B-chain signal peptides are probably removed co-translationally because the precursor proteins do not begin with the signal but with the pyroglutamic acid residue of either AKH I for the A-chain, or AKH II for the B-chain. Cyclization of the translated glutamine into pyro-glutamate appears to be a very early step in the AKH biosynthetic pathway and may also be co-translational.

To our surprise pulse-chase experiments on CC showed that the A-and B-chains are not the immediate precursors of AKH I and AKH II respectively. The immediate antecedents of the AKHs and the APRPs are in fact dimeric proteins constructed from the A- and B-chains. These constructs occur in all three possible forms which we call P1(A + A), P2(A + B) and P3(B + B). P1 is therefore a precursor of AKH I and a homodimer of the α- chain, which we call APRP 1. P2, the heterodimeric construct, is an AKH I and an AKH II precursor and precursor of APRP 2, a heterodimer of the α- and β-chains. P3, a homodimer of the B-chain, is precursor to AKH II and the homodimer of the α-chain or APRP 3. The formation of the dimeric constructs by oxidation of the single cysteine residue in the A and B chains appears to be an extremely rapid process because vanishingly little of the independent A- and B-chain monomers can be detected in the CC.

To summarize the early steps in biosynthesis of the AKHs and APRPs, two mRNAs are translated in all cells of the CC glandular lobes producing prepro-AKH I (signal + A-chain) and prepro-AKH II (signal + B-chain). Early post or co-translational steps involve removal of the signal peptides, cyclization of the N-terminal glutamines of the A and B-chains and oxidation of cysteine residues to form the three possible dimers of the A and B-chain sub-units. These events, which produce P1, P2 and P3, appear to be extremely rapid in comparison to the slow rate of transformation of the P dimers into the five accumulating products--AKH I, AKH II and the three dimer APRPs. Complete processing of newly formed Ps takes about 7 hours. The difference in the kinetics of the two stages of AKH biosynthesis, suggests that the early post-translational events occur in the endoplasmic reticulum very close to the sites of protein synthesis, and secondary events leading to the enzymatic processing of the AKHs and APRPs occur in a separate compartment.

The formation of dimer precursors and the subsequent processing events have been studied directly using *in vitro* culture methods and the pulse-chase experimental paradigms. Moreover the structural chemistry, including protein sequencing and sizing, were performed on precursor and products (APRPs) isolated and purified from the CC following their identification by pulse-chase experiments. The molecular features of AKH biosynthesis we discovered could not have been inferred from the sequence of cDNA or genomic clones. Such nucleotide sequences give deduced proteins and not the structures of the precursors explicitly. Special features of the CC allowed us to determine rather than deduce the precursor structures. Evidence for their dimeric forms was obtained by performing size exclusion chromatography and protein sequencing on purified precursor in reduced and native form. The dimeric structures could not have been inferred from the sequence of the cDNA alone, neither could the subsequent processing events (see below).

Pro-AKH I (A-chain) and pro-AKH II (B-chain) contain the AKH sequences separated by classical processing sites from the 28 residue α and β peptides. The use of these sites in

processing could perhaps have been correctly inferred from the nucleotide sequences of cDNA clones alone. However, the α and β peptide sequences contain additional potential processing sites (one dibasic and a single argenine, see Fig. 3) and their presence might have suggested additional peptides are produced by enzymatic processing within the α and β sequences. We know from protein sequencing however that these 28 residue sequences are not cut but remain intact and are present as the three dimeric constructs of APRP 1, 2 and 3. None of this could have been inferred from the cDNA sequences.

The unexpected discovery of prohormones formed from two subunits led us to question the precise mode of biosynthesis of the dimers. There are two ways to synthesize a dimeric protein such as P_2 - (i) from proteins containing more than one copy of the monomer sequence, or (ii) from the oxidation of independently translated monomers. We could not distinguish between these alternatives by performing pulse-chase experiments because we could not detect potential precursor proteins large enough to contain more than one monomer chain and we have not convincingly demonstrated the existence of independent monomer chains prior to dimer formation. Thus on the basis of our cell biological and biochemical experiments, we could not decide between the two formal possibilities for the synthesis of the dimer precursors. This question was addressed using cell-free *in vitro* translation of CC mRNA and DNA sequencing of positive cDNA clones (Schulz-Aellen et al, 1989).

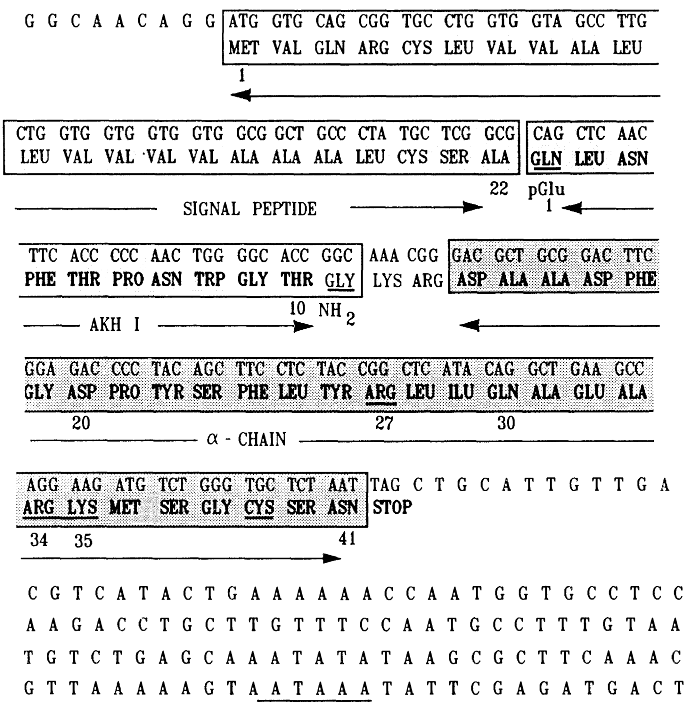


Fig. 3. Nucleotide sequence of mRNA-A encoding a signal peptide AKH I, a processing site and the α-chain of APRP 1 and APRP 2. This inferred protein sequence derived from a cloned cDNA gives the structure of prepro AKH I. The inferred 41 amino acid sequence starting from Gln is exactly that expected from protein sequencing of the P1 precursor of AKH I. (Figure modified from Schulz et al, 1989). For further explanation see text.

By translating mRNA derived from the CC *in vitro* we were able to determine the size of the complete translational protein from A-chain encoding mRNA. The size of this protein indicated that it could not accommodate more than one copy of the A-chain. Its size (6.8 kDa) is consistent with it containing one A-chain sequence plus a signal peptide. A minimal size for a protein which contains the A-chain twice, for example, is 2 x A-chain plus the 22

residue signal peptide. This gives a molecular weight of 9 kDa--too large to be the *in vitro* translational product. The identity of the *in vitro* translated protein was established by showing that its translation is blocked in the presence of an oligonucleotide which would specifically complement A-chain encoding mRNA. This experiment (see Figure 3 of Schulz-Aellen et al, 1989) indicated strongly that CC mRNA encodes a protein which contains one copy of the A-chain and that P1 is therefore produced by the oxidation of independent copies of the A-chain rather than by post-translational modification of a larger protein containing more than one copy of the A-chain. Similar experiments have indicated that the heterodimer P2 is also produced by the oxidation of independently translated proteins and that there is no protein which contains both the A- and B-chain sequences.

Confirmation that the dimeric precursors are produced from independently translated monomers is provided in sequence of cDNA clones derived from a CC cDNA library. The sequence of the cDNA clone which translates exactly into the A-chain is given in Figure 3. The inferred protein has a molecular weight exactly corresponding to the molecular weight of the protein produced during *in vitro* translation.

During cell-free *in vitro* translation, normal post-translational or co-translational modifications do not occur. So cell-free translation gives the product of genetic information alone, all additional biosynthetic events must therefore depend upon the integrity of the cells and must therefore be studied within the CC. The first event which we can infer directly from a cellular biochemical experiment is that the association between the A and B monomers is an entirely random process in the endoplasmic reticulum. Random coupling of A- and B-chain monomers would produce predictable ratios of P1, P2 and P3 dependent only on the relative amounts of the A- and B-chains present. The ratios of A- and B-chains cannot be measured directly, but because they contain one copy each of AKH I or II, they can be deduced from the relative amounts of AKH I and II. The deduced relative amounts of A and B allow us to calculate expected ratios of the dimer precursors, assuming random coupling. Actual amounts and rates of synthesis of the dimer precursors can be measured directly in pulse-labelling experiments and so the random-coupling hypothesis can be tested. Theoretical calculations and experimental measurements indicate that the predicted amounts of P1, P2 and P3 are very close to the observed amounts. Different ratios of the AKHs and APRPs can therefore be precisely regulated in the CC by differential regulation of translation of the A-chain and B-chain.

CONCLUSIONS AND FUTURE DIRECTIONS

Regulatory mechanisms, of the type mentioned above, acting on hormone biosynthesis are an important area for future investigation. We know that very strong regulatory influences on biosynthesis are exerted during postembryonic development (Hekimi and O'Shea, in preparation). For example, the relative amounts of the various precursors and products in the CC change systematically throughout postembryonic development. At early stages, during the first larval instar, AKH I and AKH II are present in approximately equal amounts, but in the adult the ratio between AKH I and AKH II reaches approximately 5:1. This regulatory influence may be at the level of transcription, translation or on subsequent post-translational events. Wherever the strong developmental regulatory influence is exerted in the pathway, we believe that the locust CC will allow us to investigate it in some detail and in a way which may not be possible in many other hormone producing systems.

Another fruitful area for investigation concerns the enzymatic mechanisms involved in post-translational processing of the pro-AKH I and pro-AKH II proteins. Many other challenging questions are raised by our model for AKH biosynthesis. For example, why is it necessary to couple monomer prohormones into dimers prior to processing? One hypothesis is that dimer structure confers a confirmation on the precursor which allows for correct processing. The existence of unused potential processing sites in the prohormone monomers suggest this possibility. This hypothesis can now be tested directly by the use of synthetic peptides, in either the dimer or the monomer form, as substrates for processing enzymes isolated from the CC.

An intriguing consequence of the participation of more than one mRNA in the formation of a prohormone is that precursors can contain more than one copy of a peptide even though that peptide is encoded only once in an mRNA. We are familiar with the idea that precursors can contain more than one copy of a particular peptide sequence, but until now it has been natural to assume that this was because that peptide sequence is encoded

more than once in the precursor's mRNA. The participation of more than one mRNA in the production of a hormone precursor is a feature of AKH biosynthesis which may not be unique. Indeed it would be surprising if the locust CC were the only tissue in which precursors are dimeric constructs. We await the investigation of other systems in sufficient detail at the level of protein chemistry to know how widespread might be the occurrence of dimeric prohormones.

ACKNOWLEDGMENTS

Supported by Swiss National Fund, UK Science and Engineering Research Council (Grant GR/E 86659) and Ciba Geigy Ltd. We thank Pat Hill for manuscript preparation.

REFERENCES

Beenakers, A.M.Th. 1969. The influence of corpus cardiacum on lipid metabolism in *Locusta migratoria*. Gen. Comp. Endocrinol. 13: Abs. 12.

Goldsworthy, G.J., Johnson, R.A. and Mordue, W. 1972a. *In vivo* studies on the release of hormones from the corpora cardiaca of locusts. J. Comp. Physiol. 79: 85-96.

Goldsworthy, G.J., Mordue, W. and Guthkelch, J. 1972b. Studies on insect adipokinetic hormones. Gen. Comp. Endocrinol. 18: 545-551.

Hekimi, S., Burkhart, W., Moyer, M., Fowler, E. and O'Shea, M. 1989. Dimer structure of a neutopeptide precursor established: Consequences for processing. Neuron 2: 1363-1368.

Hekimi, S., and O'Shea, M. 1987. Identification of precursors of the insect neuropeptide adipokinetic hormone. J. Neurosci. 7: 2773-2784.

Hekimi, S. and O'Shea, M. 1989a. Antisera against AKHs and AKH precursors for experimental studies of an insect neurosecretory system. Insect. Biochem. 19: 79-83.

Hekimi, S. and O'Shea, M. 1989b. Biosynthesis of Adipokinetic Hormones (AKHs): Further characterization of precursors and identification of novel products of processing. J. Neurosci. 9: 996-1003.

Jaffe, H., Raina, A.K., Riley, C.T., Fraser, B.A., Holman, G.M., Wagner, R.M., Ridgway, R.L. and Hayes, D.K. 1986. Isolation and primary structure of a peptide from the corpora cardiaca of *Heliothis zea* with adipokinetic activity. Biochem. Biophys. Res. Commun. 135: 622-628.

Krogh, I.M. and Norman, T.C. 1977. The corpus cardiacum neurosecretory cells of *Schistocerca gregaria*. Electron microscopy of resting and secreting cells. Acta. Zoo. 58: 69-78.

Mayer, R.J. and Candy, D.J. 1969. Control of haemolymph lipid concentration during locust flight: An adipokinetic hormone from the corpora cardiaca. J. Insect Physiol. 15: 611-620.

Orchard, I. 1987. Review: Adipokinetic hormones--an update. J. Insect Physiol. 33: 451-463.

Orchard, I. and Loughton, B.G. 1981a. The neural control of release of hyperlipaemic hormone from the corpus cardiacum of *Locusta migratoria*. Comp. Biochem. Physiol. 68A: 25-30.

Orchard, I., and Loughton, B.G. 1981b. Is octopamine a transmitter mediating hormone release in insects? J. Neurobiol. 12: 143-153.

O'Shea, M. 1985. Neuropeptides in insects: Possible leads to new control methods. pp. 133-151 in: "New Approaches in Insect Control." von Keyseringk, H., ed. Springer-Verlag, Berlin.

O'Shea, M. 1986. Insect neuropeptides: Pure and applied. pp. 3-27 in: "Proceedings of USDA International Conference on Insect Neurochemistry and Neurophysiology." Borkovec, A. B. and Gelman, D. B., eds. Humana Press, Clifton, New Jersey.

O'Shea, M., Witten, J. and Schaffer, M. 1984. Isolation and characterization of two myoactive neuropeptides: Further evidence for an invertebrate peptide family. J. Neuro-science. 4: 521-529.

Rademakers, L.H. P.M. and Beenakkers, A.M.Th. 1977. Changes in the secretory activity of the glandular lobe of the corpus cardiacum of *Locusta migratoria* induced by flight. A quantitative electron microscope study. Cell Tiss. Res. 180: 155-171.

Scheller, R.H., Jackson, J.F., McAllister, L.B., Schwartz, J.H., Kandel, E.R. and Axel, R. 1982. A family of genes that codes for ELH, a neuropeptide eliciting a stereotyped pattern of behavior in *Aplysia*. Cell 28: 707-719.

Scheller, R.H., Jackson, J.F., McAllister, L.B., Rothman, B .S., Mayei, E. and Axel, R. 1983. A single gene encodes multiple neuropeptides mediating a stereotyped behavior. Cell 32: 7-22.

Schulz-Aellen, M.-F., Roulet, E., Fischer-Lougheed, J. and O'Shea, M. 1989. Synthesis of a homodimer neurohormone precursor of locust adipokinetic hormone studied by *in vitro* translation and cDNA cloning. Neuron 2: 1369-1373.

Siegert, K., Morgan, P. and Mordue, W. 1985. Primary structures of locust adipokinetic hormones II. Biol. Chem. Hoppe-Seyler 366: 723-727.

Siegert, K.J., Morgan, P.J. and Mordue, W. 1986. Isolation of hyperglycaemic peptides from the corpus cardiacum of the American cockroach, *Periplaneta americana*. Insect. Biochem. 16: 265-371.

Stone, J.V. and Mordue, W. 1979. Isolation of granules containing adipokinetic hormone from the corpora cardiaca by differential centrifugation. Gen. comp. Endocr. 39: 5434-547.

Stone, J.V., Mordue, W., Batley, K.E. and Morris, H.R. 1976. Structure of locust adipokinetic hormone, a neurohormone that regulates lipid utilization during flight. Nature Lond. 263: 207-211.

Walter, P. and Blobel, G. 1981. Translocation of proteins across the endoplasmic reticulum. II. Signal recognition protein (SRP) mediates the selective binding to microsomal membranes of *in-vitro*-assemble polysomes synthesizing secretory protein. J. Cell Biol. 91: 551-556.

Witten, J.L., Schaffer, M.H., O'Shea, M., Cook, J.C., Hemling, M.E. and Rinehart, K.L. 1984. Structures of two cockroach neuropeptides assigned by fast atom bombardment mass spectrometry. Biochem. Biophys. Res. Commun. 124: 350-358.

Ziegler, R., Eckart, K., Schwarz, H. and Keller, R. 1985. Amino acid sequence of *Manduca sexta* adipokinetic hormone elucidated by combined fast atom bombardment (FAB) tandem mass spectrometry. Biochem. Biophys. Res. Commun. 133: 337-342.

ISOLATION AND IDENTIFICATION OF NEUROHORMONES FROM *Manduca sexta*

David A. Schooley[1], Robert L. Carney[2], Hiroshi Kataoka, Steven J. Kramer, Jorge P. Li, Anne Toschi, and Ruth Troetschler

Sandoz Crop Protection, Palo Alto, CA 94304

[1]Dept. of Biochemistry, University of Nevada, Reno, Nevada, 89557, USA
[2]Dept. of Agricultural Chemistry, University of Tokyo, Tokyo 113, Japan

INTRODUCTION

Recently we isolated and identified an eclosion hormone from heads of pharate adult *Manduca sexta*, and published these results (Kataoka et al., 1987) simultaneously with a report from Truman's group (Marti et al., 1987). Both groups reached the same conclusion; the eclosion hormone is a 62-amino acid peptide with three disulfide bridges. Subsequently we used the same isolation scheme with some modifications to isolate and identify a diuretic hormone (Kataoka et al., 1989b) and an allatotropin (Kataoka et al., 1989a) from *M. sexta* heads. The sequence of only one other eclosion hormone, that of *Bombyx mori*, is currently known (Kono et al., 1987). A diuretic hormone has been identified from *Locusta migratoria* of a substantially different peptide family (Proux et al., 1987). However, this is the first allatotropin identified from any insect species. In this paper we discuss the strategy used for the isolation including separation and bioassay techniques, the chemical sequences obtained, and some aspects of the hormonal role of these peptides.

HISTORICAL PERSPECTIVES ON NEUROHORMONE ISOLATION

Proctolin was the first insect neuropeptide to be isolated (Brown and Starratt, 1975) and identified (Starratt and Brown, 1975). The amount of tissue used, 125 kg, was quite large because of the minute content of this peptide and the relative insensitivity of peptide sequencing procedures then available. The 11-step isolation procedure gave an estimated recovery of 12%. This landmark work was done before the routine use of modern liquid chromatographic (LC) procedures for peptide isolation. Based on the recovery of biological activity, the amount of proctolin per cockroach corresponds to about 2 pmol per animal.

A year later the adipokinetic hormone was identified from two species of locust (Stone et al., 1976). The isolation scheme was much simpler than for proctolin, due to the use of dissected lobes of the corpora cardiaca as a source and due to the high content of adipokinetic hormone per locust (100-250 pmol, about two orders of magnitude higher than the proctolin content of a roach). Only two chromatographic steps were utilized, even without access to modern LC methods. Unfortunately, the ease of isolation of adipokinetic hormone is undoubtedly the exception rather than the rule, and many researchers may be misled from these studies, to anticipate that isolation of neuropeptides with different types of activity will be as facile as for adipokinetic hormone, rather than as difficult as for proctolin.

Bombyxin (4K prothoracicotropic hormone (PTTH)) was the first morphogenetic insect neurohormone to be identified, and is an even more graphic example of the difficulty of isolating insect neuropeptides than the isolation of proctolin. While the latter is a small,

hydrophilic peptide, bombyxin is large and relatively hydrophobic. Nagasawa et al. (1984) used a 15-step isolation procedure to obtain pure bombyxin from 648,000 *Bombyx mori* heads (4.86 kg). Correcting for ~8% recovery of biological activity, the 11 nmol of hormone obtained reflects a content of only 0.2 pmol of PTTH per head, an order of magnitude lower than proctolin content in *Periplaneta americana*.

An important problem in isolating neuropeptides is the possibility of degradation by proteases during isolation. For example, to minimize degradation in the isolation of proctolin, tissue was extracted with 7% $HClO_4$, an extremely acidic and denaturing solvent, while for PTTH, extracts were boiled to denature proteins. There is currently little knowledge on metabolism of insect neuropeptides. However, proctolin has been shown to have a half-life in *P. americana* tissue homogenates of only <1 to ~2 min at 5 nM *in vitro* (Quistad et al., 1984) or <5 min at the much higher, pharmacological concentration of 20,000 nM *in vivo* in *P. americana* (Starratt and Steele, 1984). Such rapid inactivation is consistent with its putative role as a neurotransmitter. On the other hand, *in vivo* metabolism studies on a cardioacceleratory factor from *P. americana*, Pea CAH-II (also known as periplanetin CC-1, M 1, and neurohormone D), have shown a half-life of ~60 min at the low concentration of 1-2 nM (Skinner et al., 1987). Thus, from the limited metabolic data available, there appears to be a difference in rate of metabolism of about two orders of magnitude between proctolin and Pea CAH-II, a member of the adipokinetic hormone family. Thus, the lack of strongly denaturing conditions in the isolation of adipokinetic hormone-like neuropeptides may not be a liability. However, use of conditions that denature proteases should ordinarily be assumed to be extremely important.

It is widely acknowledged that research in insect neurohormones has substantially lagged behind the rapid advances made in the last decade with mammalian systems. The fundamental advances in peptide isolation and sequencing have been applied first in mammalian systems. A number of reviews of isolation methodology exist (Esch et al., 1983) and (Wilson, 1988). We based our schemes for isolating neurohormones from *M. sexta* predominantly on procedures used for isolating vertebrate neurohormones, especially the principles outlined by Esch et al. (1983). Prior to our studies, the only neurohormone identified (Ziegler et al., 1985) from *M. sexta* was its adipokinetic hormone, pGlu-Leu-Thr-Phe-Thr-Ser-Ser-Trp- $GlyNH_2$.

CHOICE OF TISSUE SOURCE FOR ISOLATION

While neuropeptides can be isolated from extracts of whole insects (Brown and Starratt, 1975), purifications are simplified if a tissue with a high content of the neuropeptide is used for the isolation. With this strategy, surgical procedures are a mechanical purification, as exemplified by the isolation of adipokinetic hormone, or the earlier isolation from dissected vertebrate hypothalami of releasing factors acting on the pituitary (Esch et al., 1983). However, it is well-documented that levels of biogenic amines and cyclic nucleotides are higher in brains of rats removed from animals sacrificed by microwave treatment vs. decapitation (for example see Mir et al., 1983). Microwave-induced heating of the tissue denatures enzymes whose role is to inactivate these chemical messengers.

While there do not appear to be reports of differences in content in tissues removed from animals with microwave treatment, it seems reasonable that considerable degradation of neuropeptides may occur in tissues during dissection. Thus, to mitigate neuropeptide proteolysis after dissection, tissues should be immediately frozen in dry ice or liquid nitrogen. A number of researchers have preferred to dissect brains or corpora cardiaca from insects, rather than using whole heads. However, microsurgery is obviously slower than slicing off an insects head directly into a container of dry ice. In isolating neuropeptides from *M. sexta*, we chose the latter approach, despite knowing it would require more chemical purification than with dissected brains.

CHOICE OF PURIFICATION STEPS

A neuropeptide isolation can be divided into the extraction step, the defatting step, the pre-LC purifications, and the LC purification steps (Esch et al., 1983). This strategy is a testament to the power and simplicity of purification of peptides by LC techniques. Reversed-phase LC, in particular, has truly revolutionized neuropeptide isolation.

Esch et al. (1983) advocate extraction of peptides from tissues with an acidic solvent, followed by partitioning the extract with an organic solvent (hexane or chloroform) to remove lipids. In our hands, during such partitions emulsions frequently form that make phase separation difficult and incomplete. The opposite order of these steps was used for isolation of bombyxin (Nagasawa et al., 1984), namely, to first extract the tissue with acetone to remove fats. Then the defatted tissue residue is extracted with an appropriate solvent to obtain neuropeptides. This sequence is more rapid and may give higher recoveries.

The majority of vertebrate neuropeptides are somewhat basic, explaining the success of extraction with acidic solvents. Typical of these procedures are extraction with 2 N acetic acid containing 10 mg/l of phenylmethylsulfonyl fluoride (PMSF, a serine protease inhibitor) and 10 mg/l of pepstatin A (an acidic protease inhibitor) (Esch et al., 1983). The activity of most enzymes is much lower at low pH; addition of protease inhibitors further reduces proteolysis during extraction. An interesting example of denaturing proteases by a combination of low pH and high salt content is the "Bennett solvent": 5% formic acid, 1% trifluoroacetic acid (TFA), and 1% NaCl in 1 M HCl (Bennett et al., 1981). However, HCl is highly corrosive to a stainless steel Polytron homogenizer; Bennett et al. used a glass homogenizer.

PRECAUTIONS USED TO AVOID LOSSES DURING ISOLATION

Peptides and proteins will bind to vessels used in common laboratory operations. The proclivity to bind is greatest for hydrophobic peptides and depends on the nature of the surface. Polypropylene has the least affinity for peptides. Glass must be used with caution, and other materials (especially polystyrene) avoided. Adsorptive losses are greatest when the sample is highly purified, as adsorption is a surface phenomenon: once the surface is saturated with sample, it will have reduced adsorptive activity. Therefore, in purifications except for the final step, we added to every fraction bovine serum albumin, in quantity greater than the peptide of interest.

A related problem is the propensity of peptides to adsorb when solvent is concentrated. In fact, Esch et al. (1983) recommend loading fractions from a purification step onto LC columns without concentration, by pumping the solution onto the LC column. If the fraction contains organic solvent from reversed-phase chromatography, it must be diluted with at least two to three times its volume of water, or the peptide may not bind to the reversed-phase column. With certain brands of LC pumps, the solution can be loaded readily using a valve built into the pump.

BIOASSAY PROCEDURES

Eclosion hormone bioassay

Eclosion hormone activity in fractions from each purification step was detected using an *in vivo* assay on pharate adults of *Heliothis virescens*, similar to other assays described previously (Fugo, 1983; Truman, 1973). Insects were injected in the dorsal thorax with 5 µl aliquots dissolved in physiological saline, approximately 7 hr before normal eclosion, which occurs just after the onset of darkness. Moths eclosing within 3 hr of injection were scored as receiving eclosion hormone, a positive response. *H. virescens* is an excellent animal for the eclosion hormone assay as its photoperiod-gated eclosion occurs over a much narrower time window than *M. sexta*.

Allatotropin assay

Throughout our purification procedure we used an *in vitro* radiochemical method for assaying the ability of test samples containing allatotropin to stimulate the secretion of juvenile hormone from corpora allata of adult female *M. sexta* 0-4 hr after eclosion. This method measures incorporation of the labeled moiety from L-[*methyl*-^{14}C]methionine into juvenile hormone in the ultimate step of its biosynthesis. We used the method of Pratt and Tobe (1974) as modified for *M. sexta* (Kramer and Law, 1980). However, instead of assaying for juvenile hormone production by chloroform extraction followed by TLC analy-

sis, we used a rapid isooctane partitioning assay for juvenile hormone (Feyereisen and Tobe, 1981).

In vivo diuretic hormone bioassay in Pieris rapae

Diuretic hormone activity in fractions from purification steps was detected using an assay with newly emerged adult *Pieris rapae in vivo*, modified from one previously reported for *Danaus plexippus* (Dores *et al.*, 1979). In addition to the application of a photoperiod-based heat pulse (Dores *et al.*, 1979), adult eclosion was further synchronized by placing pharate adults in a cardboard container seated on a warm water bath (≤45 °C). Newly eclosed adult butterflies were anesthetized with CO_2 within 1 min of the molt. They were then neck ligated within 15 min, the head was immediately removed, and the wound sealed with melted wax. Each animal was suspended from the side of a 1 oz plastic cup until the wings expanded. They were weighed within 1 hr of the molt. Untreated controls were weighed about 15 min after eclosion, but not otherwise treated.

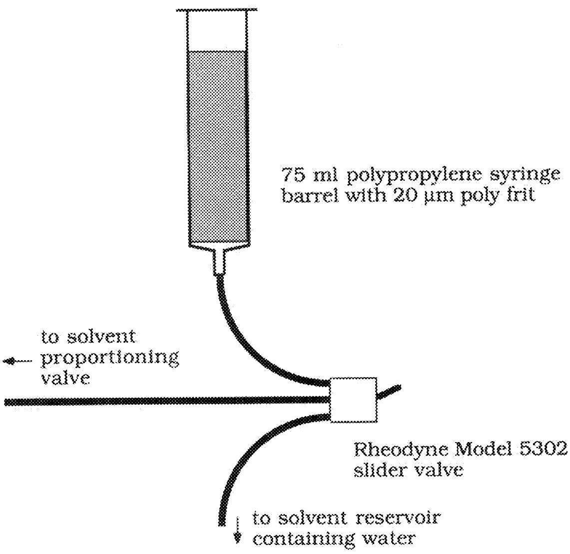


Fig. 1. Apparatus devised for loading water-diluted fractions from reversed-phase LC onto a column for subsequent LC separation. A low pressure slider valve is used before the pump in the liquid chromatograph to select flow (normally from the water reservoir) to a polypro-pylene reservoir holding the diluted solution. Hundreds of milliliters of diluted solution can be loaded onto a column with this device.

In vivo diuretic hormone bioassay in Manduca sexta

Wandering fifth stadium larvae of *M. sexta* lose ~30% of their body weight during the 48 hr period which precedes pupation. Pre-wandering, post-feeding *M. sexta* were used just after lights out (12-12 photoperiod). To assay for activity of synthetic diuretic hormone, these larvae were weighed, then injected with 25 µl *M. sexta* saline or 25 µl saline containing 0.1 mg synthetic diuretic hormone. After 4 hr, each larva was again weighed and the dry weight of frass was subtracted to determine net weight of water lost.

Preparation of samples for bioassay

It is necessary during many purification steps to utilize buffers, salts or acids, whose presence in fractions will be incompatible with most bioassays. For all of our reversed-

phase LC steps, we utilized volatile ion-pairing reagents, TFA or heptafluorobutyric acid (HFBA), so that aliquots for bioassay could be completely dried down in a vacuum centrifuge. However, the salts used in our preliminary SP-Sephadex ion exchange separation, and the ion exchange LC purification step, had to be removed prior to assay. We utilized commercially available empty reservoirs for solid-phase extraction cartridges, filled with 100 mg of bulk Vydac C_4 packing (20-30 µm particle diameter), because solid phase extraction cartridges are not commercially available packed with wide-pore supports suitable for proteins. Aliquots of fractions were loaded onto the cartridges with aqueous buffer, washed with 0.1% TFA to remove salts, and then the peptides eluted with 60% acetonitrile in 0.1% aqueous TFA. The organic-containing effluent from the cartridge was evaporated in a vacuum centrifuge, but only after adding ~100-250 µg of bovine serum albumin prior to dry-down to mitigate the adsorption of peptides. Each cartridge was generally used for a number of samples, provided it was properly re-equilibrated with aqueous buffer after eluting the prior sample with 60% acidic acetonitrile.

***M. sexta* Eclosion Hormone Isolation Scheme**

10,000 trimmed heads from pharate adults (420 g)

↓ 1) Homogenize with acetone, filter (delipidation)

↓ 2) Extract with strongly acidic buffer containing protease inhibitors

↓ 3) SP Sephadex chromatography; EH in 0.05 M NH_4OAc, pH 7.0

↓ 4) Vydac C_4 cartridge; EH in 35% CH_3CN/0.1% TFA fraction

↓ 5) Vydac C_4 semiprep LC, 15-30% 1-propanol/0.1% TFA gradient

↓ 6) " " " , 20-40% CH_3CN/0.1% TFA gradient

↓ 7) TSK DEAE-5PW LC, 0-0.5 M NaCl/0.02 M Tris·HCl, pH 8.0 gradient

↓ 8)Vydac C_4 analytical LC, 30-45% CH_3CN/0.1% HFBA gradient

~1.7 nmol pure EH (~12 µg); purification factor 35 x 10^6 from heads

0.17 pmol/head

Fig. 2. Scheme for isolation of an eclosion hormone from heads of *Manduca sexta*, based on data from (Kataoka et al., 1987).

AN ISOLATION SCHEME FOR *Manduca sexta* NEUROPEPTIDES

We recently isolated the eclosion hormone (Kataoka et al., 1987), an allatotropin (Kataoka et al., 1989a), and the diuretic hormone (Kataoka et al., 1989b) of *Manduca sexta* using slight variants of a very similar procedure, requiring only eight or nine steps from "trimmed" heads. As this isolation scheme is relatively simple and possibly of more general utility, we describe its crucial features.

Eclosion hormone was the first *M. sexta* neurohormone isolated with this procedure (Fig. 2). Subsequently, we attempted to isolate diuretic hormone from side fractions of the eclosion hormone isolation. Unfortunately, diuretic activity was diminished or lost when we evaporated the relatively large amounts of solvent from either the reversed-phase cartridge step, or the semipreparative purifications, possibly due to adsorption or oxidation. We had hoped that the large amounts of other proteins or peptides at these earlier stages of the purification would afford protection to the diuretic hormone, and had not experienced serious losses of eclosion hormone at comparable steps. When fraction size is small, as from an analytical-scale (4.6 mm or less i.d.) column, fractions can be diluted with water and loaded on the column for the next purification by repetitive injections with a 5 ml loop. However, for the larger volumes from the C_4 cartridge or semi-preparative columns, we were forced to modify the factory version of the Perkin-Elmer Model 410 BIO pumping system so that water-diluted fractions containing organic solvent could be loaded onto the column via the pump. The apparatus we devised (Fig. 1) consists of a switching valve to

select a suitable reservoir instead of the aqueous buffer reservoir. The diluted fraction is loaded into a 75 ml polypropylene syringe barrel. It is desirable to slightly pressurize this reservoir with helium (~0.5 atm) so that the solution is fed to the pump under slight pressure, as it is difficult to avoid introducing air bubbles into the pump using this technique.

The starting material for isolating the hormones was 10,000 heads of pharate adult *M. sexta*, which were cut off directly into dry ice. A 5 mm cork borer was used to "trim" a frozen tissue portion containing the brain and corpus allatum/corpus cardiacum complex away from the eyes, proboscis, antennae, and other parts of the head. The trimmed heads (0.42 kg) were homogenized in acetone (Brinkmann Polytron) to remove lipids. The extract was filtered, the acetone discarded, and the residue re-extracted with an acidic solvent with protease inhibitors: 1 M acetic acid containing 20 mM HCl, 0.1 mM PMSF, and 0.01 mM pepstatin A. As PMSF is hydrolytically unstable, this solvent must be made up just before use. Both protease inhibitors are poorly soluble in water, and were dissolved in the acetic acid required for the 1 M final concentration, then diluted into water. The extract was centrifuged, the supernatant decanted, and the pellet re-extracted. All steps were performed at 0-4 °C. The combined acidic supernatants were loaded onto an SP-Sephadex C-25 column in the cold room, which was eluted sequentially with 1 liter each of 1 M acetic acid and 50 mM NH_4OAc (pH 4), followed by 50 mM, 100 mM, 200 mM, 400 mM, and 800 mM NH_4OAc (each at pH 7). Aliquots from each fraction from the step elution were desalted as described above; eclosion hormone eluted in the 50 mM NH_4OAc fraction, allatotropin in the 100 and 200 mM NH_4OAc fractions, and diuretic hormone in the 400 and 800 mM NH_4OAc.

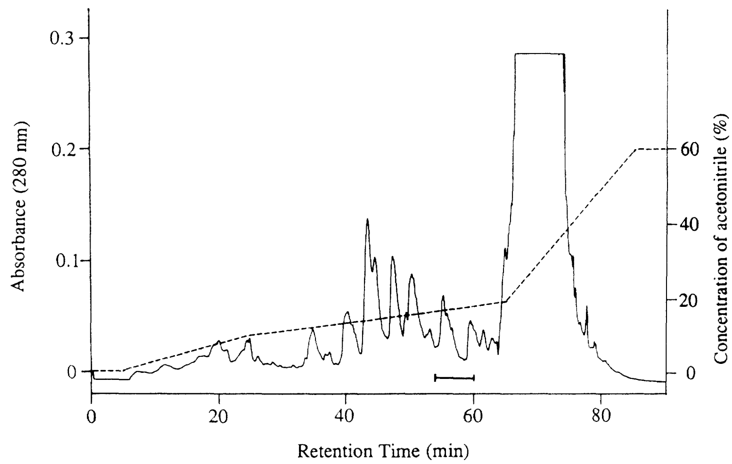


Fig. 3. First semi-preparative isolation of *M. sexta* allatotropin on a 25 x 1.0 cm Vydac C_4 column. The solid line indicates absorbance at 220 nm, while the dashed line indicates the composition of the acetonitrile/0.1% TFA gradient. The biologically active fractions eluting from 54-60 min are marked with a bar. A very slow gradient was used in the area where the biological activity eluted, while a fast gradient was used to elute massive amounts of UV-absorbing impurities. Fine-tuning a gradient in this way requires several pilot experiments, but is generally invaluable at the early stages of isolation (Kataoka et al., 1989a).

The large volumes of buffer were concentrated and desalted by applying them to a cartridge column, 10 g of Vydac C_4 bulk packing contained in a 75 ml polypropylene syringe barrel (a scale-up of the desalting procedure described in the Bioassay procedures). The solution was pulled through by vacuum, and the cartridge washed with 100 ml of 0.1% TFA. All fractions from SP-Sephadex contained varying amounts of pigments, most of which were not retained on the Vydac C_4. The desalted peptides could then be eluted using step gradients of acetonitrile/water containing 0.1% TFA to provide some enrichment, or the column could simply be eluted with 60% acetonitrile/0.1% aqueous TFA to elute all

peptides together. We used 100 ml each of 20%, 35%, and 60% acetonitrile/0.1% TFA to elute eclosion hormone and diuretic hormone. Both hormones were recovered in the 35% acetonitrile/0.1% TFA fraction, freeing them of both very hydrophilic and very hydrophobic peptides. Allatotropin was eluted together with other peptides using 60% acetonitrile.

For eclosion hormone, we concentrated the solvent after the C_4 cartridge chromatography and semipreparative chromatography without evidence of loss of biological activity. However, on isolation of diuretic hormone and allatotropin, the active organic fraction was diluted with twice its volume of water and pumped onto a 25 x 1.0 cm Vydac C_4 semipreparative column as shown in Fig. 1. The pump and column were equilibrated with 0.1% TFA prior to loading. The choice of solvent and ion pairing reagent for the first semipreparative separation can be crucial, as we found that massive amounts of protein, perhaps structural proteins from cuticle, elute at certain concentrations of organic modifier. For eclosion hormone we used a gradient of 1-propanol/0.1% TFA for the first semi-prep

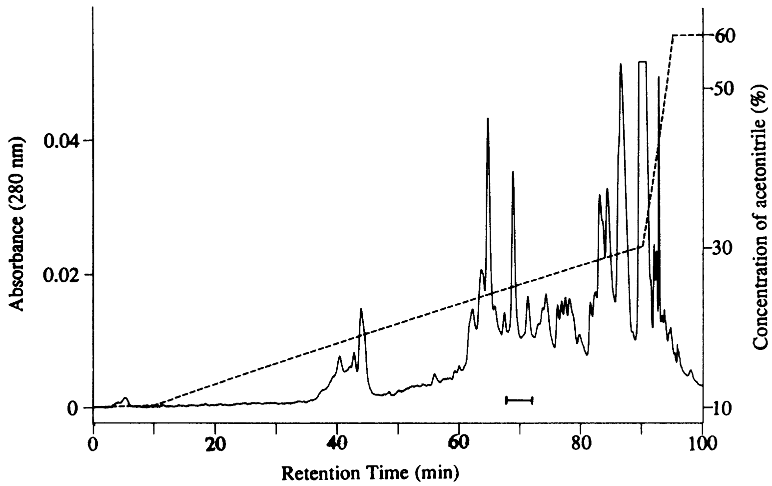


Fig. 4. Second semi-preparative isolation of *M. sexta* allatotropin. The same column was used as in Fig. 3, but HFBA was used instead of TFA as ion-pairing reagent. The solid line indicates absorbance at 220 nm, while the dashed line shows the percentage of acetonitrile. The biologically active fractions elute from 68-72 min (solid bar with tics), in a region of the chromatogram with relatively few constituents.

LC step, to provide a different selectivity from acetonitrile elution of the C_4 cartridge. For both allatotropin and diuretic hormone, we used an acetonitrile/0.1% TFA gradient for the first semi-preparative LC step. With allatotropin, biological activity was recovered in a zone which corresponded to about 17-19% acetonitrile on the gradient (see Fig. 3).

Active fractions from the first semipreparative RPLC purification were pooled, diluted with twice the volume of water, and again pumped onto the semi-preparative column. For each hormone, the second semipreparative step used a different mobile phase from the prior step. For allatotropin, this was a 10-30% acetonitrile/0.1% HFBA gradient; biological activity was recovered in fractions corresponding to 24.5-25.5% acetonitrile (Fig. 4, 6-7% higher than when TFA was the ion pairing reagent). For diuretic hormone, we utilized 10-30% 1-propanol/0.1% TFA for the second semi-prep isolation; for eclosion hormone it was 20-40% acetonitrile/0.1% TFA.

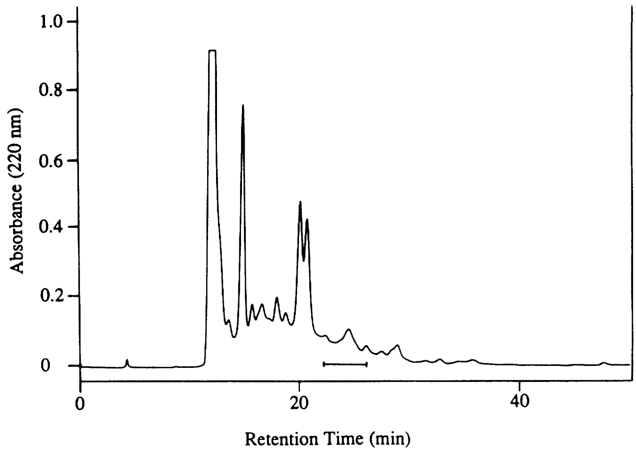


Fig. 5. Separation of active fraction from Fig. 4 on an ion exchange LC column (Toyo Soda TSK SP-5PW), eluted with a gradient of 0 to 0.5 M NaCl in a 20 mM sodium phosphate buffer, pH 6.25. The active fraction eluted from 22-26 min (shown by solid bar with tics), which corresponded to about 0.09-0.10 M NaCl in the gradient. Buffers contained 10% acetonitrile to retard microbial growth.

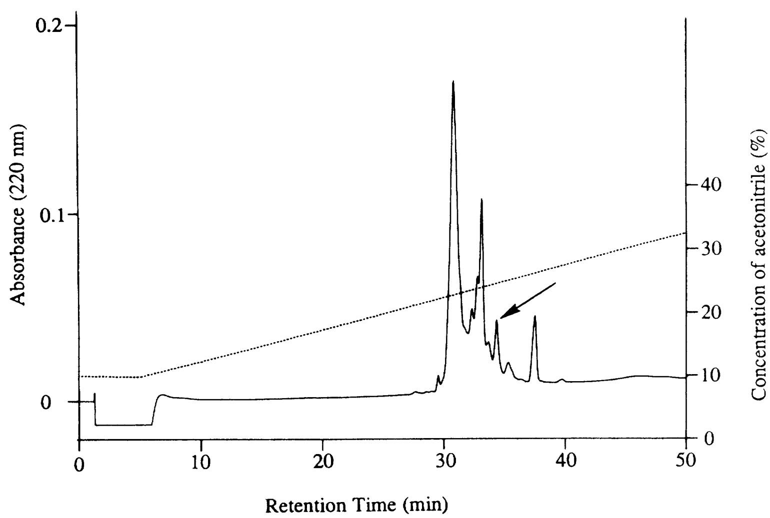


Fig. 6. Separation of active fraction from Fig. 5 on analytical RPLC (15 x 0.46 cm Vydac C_{18} column). The solid line indicates absorbance at 220 nm; the dashed line shows the percentage of acetonitrile in the gradient. The ion pairing reagent was TFA as in Fig. 5. The biologically active peak used for sequence and amino acid analysis is shown with an arrow.

The subsequent step was an ion exchange LC separation. For allatotropin (Fig. 5) and diuretic hormone, we used a TSK SP-5PW analytical column, equilibrated with 0.02 M sodium phosphate buffer, pH 6.25, eluted with a gradient of sodium chloride from 0-0.5 M. For the more acidic eclosion hormone, we used an anion exchange column, TSK DEAE-5PW, and pH 8.0 Tris-HCl buffer. These useful columns are packed with hydrophilic polymers with minimal adsorptive properties (unlike polystyrene polymers).

The active fractions from ion exchange LC purification of eclosion hormone were loaded directly onto a Vydac C_4 analytical column (15 x 0.46 cm) and eluted with an acetonitrile/0.1% HFBA gradient. Pure eclosion hormone suitable for sequence analysis was obtained at this step. The same procedure and mobile phase, but a somewhat different gradient, was used for diuretic hormone after the ion exchange step. However, the diuretic hormone still appeared slightly impure and was purified on a 25 x 0.21 cm Vydac C_4 microbore column with acetonitrile/0.1% TFA gradient.

The active fraction of allatotropin from ion exchange LC was loaded onto a Vydac C_{18} analytical column (15 x 0.46 cm) eluted with a gradient of 10-40% acetonitrile/0.1% TFA. A number of peaks eluted rather close together, with one biologically active peak (arrow in Fig. 6) chromatographing in the tail of several other peaks at a retention time of 34.3 min, corresponding to ~17% acetonitrile. This is an example of a step where the column packing was changed, but the mobile phase was one used earlier (in the fifth step, Fig. 3). While the impurities are rather close eluting, the peak indicated was sufficiently pure for complete sequence analysis of the 13-residue peptide, H-Gly-Phe-Lys-Asn- Val-Glu-Met-Met-Thr-Ala-Arg-Gly-Phe, using only a 10% aliquot of the pure material.

Using these highly similar schemes, about 1.7 nmol (~12 µg) of eclosion hormone, about 5 nmol (~24 µg) of diuretic hormone, and about 1.5 nmol (~2.2 µg) of allatotropin were recovered from 10,000 heads. Because the bioassays are at best semi-quantitative, we made no attempt to determine the percentage recovery of biological activity. Assuming high recoveries (for which we have no evidence) this would correspond to about 0.17, 0.5, and 0.15 pmol/head each of eclosion hormone, diuretic hormone, and allatotropin, respectively. This is similar to the recovery of 4K PTTH from *Bombyx*. The purification factor from heads ranges from 18 to 190 x 10^6. Considering that the first two steps probably have a rather small purification factor, this is a remarkable testament to the power and resolution of LC techniques for peptide purification.

ECLOSION HORMONE SEQUENCE AND BIOLOGICAL PROPERTIES

The primary amino acid sequence of *M. sexta* eclosion hormone has now been established independently by two groups (Kataoka et al., 1987; Marti et al., 1987) using automated Edman degradation of the reduced, carboxymethylated hormone and peptide fragments thereof (Fig. 7). Recently these results were confirmed from the sequence of the gene (Horodyski et al., 1989). Kataoka et al. (1987) cleaved the hormone with endoproteinases Lys-C or Glu-C, separated each digest, and sequenced the fragments. Marti et al. (1987) used a cyanogen bromide cleavage and endoproteinase Lys-C to generate fragments for sequencing. Eclosion hormone proved to be a rather difficult peptide to sequence, especially as regards the C-terminus. Both groups had to resort to a third cleavage to obtain a suitable C-terminal fragment. Kataoka et al. (1987) cleaved a mixture of two Glu-C fragments with proline-specific endopeptidase to give a pentapeptide, (Phe-Leu-Asn-Lys-Leu). Marti et al. (1987) performed a specific acid cleavage of eclosion hormone, affording the 11 residue peptide Phe-Ala-Ser-Ile-Ala-Pro-Phe-Leu-Asn-Lys-Leu. Kataoka et al. (1987) additionally isolated two single amino acids from endoproteinase Lys-C cleavage; Lys and Leu, which they converted to phenylthiocarbamyl derivatives and analyzed by LC. This analysis revealed that the Leu from residue 62 existed in the free acid, rather than amidated, form. This result was confirmed from the gene sequence, as there is a stop signal immediately after the Leu^{62}, whereas an amidated form would require a Gly residue following the Leu. The gene contains two introns, one in a non-coding region and the other between amino acid residues 4 and 5, but is rather simple in coding for a signal peptide attached to a single copy of the eclosion hormone sequence, and the termination signal (Horodyski et al., 1989).

The sequence of eclosion hormone from *Bombyx mori* was reported (Kono et al., 1987) shortly before that of *M. sexta*; it has only 80% sequence identity and is 61 residues long, terminating in a Lys. It is not currently known if this is the complete sequence. Of the twelve residues that differ between the two hormones, three would require two base changes in the codon. This seems to reflect a greater degree of evolution of the gene between these two lepidopterans than might be expected from the relatively highly conserved adipokinetic hormone family of peptides, in which case sequences are known from Orthoptera, Dictyoptera, Lepidoptera, Coleoptera, and Diptera. Computer searches of protein databases have shown no significant degree of sequence similarity of either eclosion hormone with any other known peptide hormone.

```
                                    10              15
H-Asn-Pro-Ala-Ile-Ala-Thr-Gly-Tyr-Asp-Pro-Met-Glu-Ile-Cys-Ile-
              20                                    30
  Glu-Asn-Cys-Ala-Gln-Cys-Lys-Lys-Met-Leu-Gly-Ala-Trp-Phe-Glu-
                                    40              45
  Gly-Pro-Leu-Cys-Ala-Glu-Ser-Cys-Ile-Lys-Phe-Lys-Gly-Lys-Leu-
              50                                    60
  Ile-Pro-Glu-Cys-Glu-Asp-Phe-Ala-Ser-Ile-Ala-Pro-Phe-Leu-Asn-
    62
  Lys-Leu-OH
```

Fig. 7. Sequence of *Manduca sexta* eclosion hormone.

As of the end of 1989, there are no reports of successful synthesis of eclosion hormone by either solid-state peptide synthesis or via recombinant DNA techniques. Chemical synthesis of linear peptides with 62 residues is challenging, but with three disulfide bonds, it is formidably difficult. The lack of availability of synthetic material has hindered further study of its physiology and the availability of antibodies. We are fortunate that a great deal is known of the physiology of eclosion hormone from the extensive studies of Truman's group over a number of years (Reynolds and Truman, 1983).

DIURETIC HORMONE SEQUENCE AND BIOLOGICAL PROPERTIES

Structural analysis

Automated Edman degradation of intact diuretic hormone (0.5 nmol, or 10% of the sample) yielded a single amino acid sequence with ~80% initial yield and ~95% repetitive yield, and residues 1-40 were assigned. Trypsin digestion of diuretic hormone (1.5 nmol) gave six fragments (T-1 to T-6, not shown), which were also sequenced. The results suggested that T-5 was a carboxyl-terminal fragment of diuretic hormone, because only T-5 had neither Arg nor Lys at its carboxyl terminus. Therefore, diuretic hormone was regarded as a 41 residue peptide.

The nature of the carboxyl terminus was established by comparison of RPLC retention times of T-5 and two synthetic hexapeptides (Asn-Phe-Leu-Asn-Asp-Ile) prepared in the amidated and free acid forms. The data showed clearly that the native T-5 coeluted with the amidated synthetic hexapeptide. Hence, the complete structure of diuretic hormone was established as shown in Fig. 8. This structure was also supported by amino acid analysis of intact diuretic hormone and its tryptic fragments.

Carp Urotensin I	N D D P P I S I D L T F H L L R N M I E M A R N E N Q R E Q A G L N R K Y L D E V-NH2
Sucker Urotensin I	N D D P P I S I D L T F H L L R N M I E M A R I E N E R E Q A G L N R K Y L D E V-NH2
Sauvagine	Q G P P I S I D L S L E L L R K M I E I E K Q E K E K Q Q A A N N R L L L D T I-NH2
Manduca sexta DH	R M P S L S I D L P M S V L R Q K L S L E K E R K V H A L R A A A N R N F L N D I-NH2
Caprine/Ovine CRF	S Q E P P I S L D L T F H L L R E V L E M T K A D Q L A Q Q A H S N R K L L D I A-NH2
Human/Rat CRF	S E E P P I S L D L T F H L L R E V L E M A R A E Q L A Q Q A H S N R K L M E I I-NH2

Fig. 8. Complete amino acid sequence of *Manduca sexta* diuretic hormone, and its similarity to the sequences of urotensin I (carp and sucker), sauvagine, and corticotropin releasing factor (CRF) from four species of mammals.

Synthesis and biological properties of Manduca sexta diuretic hormone

We synthesized *M. sexta* diuretic hormone, as well as an analogue with an acid at the carboxyl terminus, using conventional automated solid phase methodologies (Barany and

Merrifield, 1980). The synthetic amidated and acidic forms were compared to native diuretic hormone by reversed-phase LC analysis, which again established the identity of native diuretic hormone with the synthetic amidated form. Synthetic diuretic hormone and its acid analogue were tested in the *P. rapae* assay. The approximate ED_{50} for the amide form was 0.1 ng per animal, that for the acid 0.1 mg per animal.

We investigated the effects of synthetic diuretic hormone on *M. sexta* larvae *in vivo*. Late fifth stadium larvae, weighing ~9 g, excrete approximately one third of their body weight in water over a 48 hr period. When 0.1 mg of synthetic diuretic hormone was injected into post-feeding, pre-wandering larvae, a mean of 226 mg of fluid was lost over a 4 hr period from those injected with synthetic diuretic hormone, while the control larvae excreted a mean of 98 mg. This is a highly significant difference ($P < 0.001$).

We checked for sequence similarity of *M. sexta* diuretic hormone with known peptides using a computer program, which located a substantial similarity with sauvagine Fig. 8. Sauvagine was isolated from skins of the frog *Hyllomedusa sauvagei* using a bioassay based on its potent *antidiuretic* effect in rats (Montecucchi et al., 1980). If allowance is made for a one-amino acid insertion of histidine in *M. sexta* diuretic hormone (position 27), 40% of the residues are identical. An additional 16 amino acids in *M. sexta* diuretic hormone could result from single-base mutations in the sauvagine gene. The location of these amino acids strengthens the supposition that histidine has been inserted in a well conserved sequence. Sauvagine is but one member of an important peptide family including corticotropin releasing factor (CRF) and urotensin I (Vale et al., 1983). CRF, isolated and identified first from sheep hypothalami (Vale et al., 1983), has been sequenced subsequently from the rat (rCRF, (Vale et al., 1983)), goat (cCRF, (Ling et al., 1984)), and cow (bCRF, (Esch et al., 1984)). The structure of human CRF was deduced from the sequence of its gene (hCRF, (Shibahara et al., 1983)). The sequence identity of *M. sexta* diuretic hormone with oCRF/cCRF is 32%; an additional 41% similarity would result from single-base changes in the gene, with slightly lower similarity to hCRF/rCRF. Urotensin I, peptides biologically and chemically homologous to sauvagine and CRF, were isolated and identified from fish urophysis. *M. sexta* diuretic hormone has 27% direct sequence identity with both white sucker and carp urotensin I (Vale et al., 1983); an additional 44% identity with sucker urotensin I could come from single base changes in the gene.

Thus, *M. sexta* diuretic hormone is the newest member of the sauvagine/CRF/urotensin I family of peptides. The most studied physiological/ pharmacological effects of this peptide family appear to be the stimulation of ACTH release and hypotensive/-vasodilatory properties; the antidiuretic effect of sauvagine has been attributed to its potent hypotensive action (Montecucchi and Henschen, 1981). The potency and nature of the effects elicited by each peptide varies with the species in which it is tested. For example, urotensin I elicits a strong and selective hypotensive/vasodilatory effect in mammals, comparable to sauvagine and much more potent than that caused by the mammalian form oCRF (Ichikawa et al., 1982). In contrast, injection of 500 pmol of urotensin I, sauvagine, and oCRF did not stimulate fluid excretion in the *P. rapae* assay (R. Troetschler, unpublished). By comparison, the ED_{50} of *M. sexta* diuretic hormone is ~0.02 pmol. We do not at this time have any data on biological activity of truncated analogues of *M. sexta* diuretic hormone, although this would clearly be of interest.

ALLATOTROPIN SEQUENCE AND BIOLOGICAL PROPERTIES

Sequence analysis of the purified allatotropin (~150 pmol, or 10% of the purified sample) was performed by automated Edman degradation on an Applied Biosystems 477A pulsed liquid phase sequencer with Model 120A phenylthiohydantoin analyzer. This analysis indicated the primary structure Gly-Phe-Lys-Asn-Val-Glu-Met-Met-Thr-Ala-Arg-Gly-Phe. Hydrolysis and amino acid analysis of the peptide revealed the amino acid composition to be in complete accord with the amino acid composition from sequence analysis. Peptides having the indicated sequence were synthesized with the carboxyl terminus in the amidated and free acid forms with standard automated solid-phase techniques. These purified peptides were analyzed by reversed-phase LC. Native allatotropin coelutes with the synthetic amidated form when coinjected, confirming the sequence analysis and proving that the carboxyl terminus is amidated. Thus, the complete structure of allatotropin is:

H-Gly-Phe-Lys-Asn-Val-Glu-Met-Met-Thr-Ala-Arg-Gly-Phe-NH_2.

A computer search of protein databases showed no significant sequence similarity to known peptide hormones from any organism.

The biological activities of the synthetic amidated and free acid forms were compared with native allatotropin. The potency of the synthetic amidated form could not be distinguished from that of native allatotropin, whereas that of the free acid form was only partially stimulatory at 100 to 1000 times higher concentrations. As this suggested that the nature of the carboxyl terminus is crucial for activity, we synthesized a series of analogues truncated from the amino terminus. Assays of the purified peptides showed that allatotropin(4-13), allatotropin(5-13), and allatotropin(6-13) had biological activity essentially indistinguishable from the complete allatotropin sequence. However, the activity of allatotropin(7-13) was diminished about tenfold at 0.2 pmol peptide. This suggests that the biologically active core is the octapeptide allatotropin(6-13), H-Glu-Met-Met-Thr-Ala- Arg-Gly-Phe-NH_2.

We tested the effects of *M. sexta* allatotropin on the corpora allata of other developmental stages of this insect, as well as on the CA of adult insects of four other species. Curiously, this allatotropin from adult *M. sexta* had no discernible effect on the rate of juvenile hormone biosynthesis of larvae or pupae of this species. The lack of effect on pupae is less surprising than the results with larvae, as pupal CA normally do not produce any juvenile hormone. Allatotropin also had no effect on the activity of CA of adult females of the beetle *Tenebrio molitor*, the grasshopper *Schistocerca nitens*, and the cockroach *P. americana*. However, *M. sexta* allatotropin stimulated juvenile hormone biosynthesis strongly in CA of the adult female Noctuid moth *H. virescens*. Thus, we conclude that the activity of *M. sexta* allatotropin may be restricted to the adult stage of the order Lepidoptera.

ACKNOWLEDGMENTS

We thank the staff of the insect culture group at Sandoz for providing animals, Marilyn Bennett for technical assistance, Blake Cesarin for determining amino acid compositions, and Dr. Alan Roter for performing computer searches of peptide homologies.

REFERENCES

Barany, G. and Merrifield, R. B. 1980. Solid phase peptide synthesis. pp. 1-284 in: "The Peptides." Gross, E. and J. Meienhofer eds. Academic Press.

Bennett, H. P. J., Browne, C. A. and Solomon, S. 1981. Purification of the two major forms of rat pituitary corticotropin using only reversed-phase liquid chromatography. Biochemistry 20: 4530-4538.

Brown, B. E. and Starratt, A. N. 1975. Isolation of proctolin, a myotropic peptide, from *Periplaneta americana*. J. Insect Physiol. 21: 1879-1881.

Dores, R. M., Dallmann, S. H. and Herman, W. S. 1979. The regulation of post-eclosion and post-feeding diuresis in the Monarch butterfly, *Danaus plexippus*. J. Insect Physiol. 25: 895-901.

Esch, F., Ling, N., Böhlen, P., Baird, A., Benoit, R. and Guillemin, R. 1984. Isolation and characterization of the bovine hypothalamic corticotropin-releasing factor. Biochem. Biophys. Res. Commun. 122: 899-905.

Esch, F. S., Ling, N. C. and Böhlen, P. 1983. Microisolation of neuropeptides. Methods Enzymol. 103: 72-89.

Feyereisen, R. and Tobe, S. S. 1981. A rapid partition assay for routine analysis of juvenile hormone release by insect corpora allata. Anal. Biochem. 111: 372-375.

Fugo, H. 1983. EH Bioassay? Appl. Ent. Zool. 18: 540-544.

Horodyski, F. M., Riddiford, L. M. and Truman, J. W. 1989. Isolation and expression of the eclosion hormone gene from the tobacco hornworm, *Manduca sexta*. Proc. Natl. Acad. Sci. USA. 86: 8123-8127.

Ichikawa, T., McMaster, D., Lederis, K. and Kobayashi, H. 1982. Isolation and amino acid sequence of urotensin I, a vasoactive and ACTH-releasing neuropeptide, from the carp (*Cyprinus carpio*) urophysis. Peptides 3: 859-867.

Kataoka, H., Toschi, A., Li, J. P., Carney, R. L., Schooley, D. A. and Kramer, S. J. 1989a. Identification of an allatotropin from adult *Manduca sexta*. Science 243: 1481-1483.

Kataoka, H., Troetschler, R. G., Kramer, S. J., Cesarin, B. J. and Schooley, D. A. 1987. Isolation and primary structure of the eclosion hormone of the tobacco hornworm, *Manduca sexta*. Biochem. Biophys. Res. Commun. 146: 746-750.

Kataoka, H., Troetschler, R. G., Li, J. P., Kramer, S. J., Carney, R. L. and Schooley, D. A. 1989b. Isolation and identification of a diuretic hormone from the tobacco hornworm, *Manduca sexta*. Proc. Natl. Acad. Sci. USA. 86: 2976-2980.

Kono, T., Nagasawa, H., Isogai, A., Fugo, H. and Suzuki, A. 1987. Amino acid sequence of eclosion hormone of the silkworm, *Bombyx mori*. Agric. Biol. Chem. 51: 2308-2308.

Kramer, S. J. and Law, J. H. 1980. Control of juvenile hormone production: the relationship between precursor supply and hormone synthesis in the tobacco hornworm, *Manduca sexta*. Insect Biochem. 10: 569-575.

Ling, N., Esch, F., Böhlen, P., Baird, A. and Guillemin, R. 1984. Isolation and characterization of the caprine corticotropin-releasing factor. Biochem. Biophys. Res. Commun. 122: 1218-1224.

Marti, T., Takio, K., Walsh, K. A., Terzi, G. and Truman, J. W. 1987. Microanalysis of the amino acid sequence of the eclosion hormone from the tobacco hornworm *Manduca sexta*. FEBS Letters. 219: 415-418.

Mir, A. K., Pallot, D. J. and Nahorski, S. R. 1983 Biogenic amine-stimulated cyclic adenosine-3', 5'-monophosphate formation in the rat carotid body. J. Neurochem. 41: 663-669.

Montecucchi, P. C., Anastasi, A., de Castiglione, R. and Erspamer, V. 1980. Isolation and amino acid composition of sauvagine, an active polypeptide from methanol extracts of the skin of the South American frog *Phyllomedusa sauvagei* skin bioassay. Int. J. Peptide Protein Res. 16: 191-199.

Montecucchi, P. C. and Henschen, A. 1981. Amino acid composition and sequence analysis of sauvagine, a new active peptide from the skin of *Phyllomedusa sauvegei*. Int. J. Peptide Protein Res. 18: 113-120.

Nagasawa, H., Kataoka, H., Hori, Y., Isogai, A., Tamura, S., Suzuki, A., Guo, F., Zhong, X., Mizoguchi, A., Fujishita, M., Takahashi, S. Y., Ohnishi, E. and Ishizaki, H. 1984. Isolation and some characterization of the prothoracicotropic hormone from *Bombyx mori*. Gen. Comp. Endocrinol. 53: 143-152.

Pratt, G. E. and Tobe, S. S. 1974. Juvenile hormones radiobiosynthesized by corpora allata of adult female locusts *in vitro*. Life Sci. 14: 575-586.

Proux, J. P., Miller, C. A., Li, J. P., Carney, R. L., Girardie, A., Delaage, M. and Schooley, D. A. 1987. Identification of an arginine vasopressin-like diuretic hormone from *Locusta migratoria*. Biochem. Biophys. Res. Commun. 149: 180-186.

Quistad, G. B., Adams, M. E., Scarborough, R. M., Carney, R. L. and Schooley, D. A. 1984. Metabolism of proctolin, a pentapeptide neurotransmitter in Insects. Life Sci. 34: 569-576.

Reynolds, S. E. and Truman, J. W. 1983. Eclosion hormone. pp. 217-233 in: "Endocrinology of Insects." Downer, R. G. H and Laufer, H., eds. Alan R. Liss, Inc., New York.

Shibahara, S., Morimoto, Y., Furutani, Y., Notake, M., Takahashi, H., Shimizu, S., Horikawa, S. and Numa, S. 1983. Isolation and sequence analysis of the human corticotropin-releasing factor precursor gene. EMBO Journal 2: 775-779.

Skinner, W. S., Quistad, G. B., Adams, M. E. and Schooley, D. A. 1987. Metabolic degradation of the peptide periplanetin CC-2 in the cockroach, *Periplaneta americana*. Insect Biochem. 17: 433-437.

Starratt, A.N. and Brown, B.E. 1975. Structure of the pentapeptide proctolin, a proposed neurotransmiter in insects. Life Sci. 17: 1253-1256.

Starratt, A.N. and Steele, R.W. 1984. *In vivo* inactivation of the insect neuropeptide proctolin in *Periplaneta americana*. Insect Biochem. 14: 97-102.

Stone, J. V., Mordue, W., Batley, K. E. and Morris, H. R. (1976) Stucture of locust adipokinetic hormone, a neurohormone that regulates lipid utilisation during flight. Nature 263: 207-211.

Truman, J. W. 1973. Physiology of Insect Ecdysis. II. The assay and occurrence of the eclosion hormone in the Chinese Oak Silkmoth, *Antheraea pernyi*. Biol. Bull. 144: 200-211.

Vale, W., Rivier, C., Brown, M. R., Spiess, J., Koob G., Swanson, L., Bilezikjian, L., Bloom, F. and Rivier, J. 1983. Chemical and biological characterization of corticotropin releasing factor. Rec. Prog. Horm. Res. 39: 245-270.
Wilson, K. J. 1988. Purification of proteins/peptides for structural studies. pp. in: "Protein/Peptide Sequence Analysis: Current Methodologies." Brown, A.S., ed. CRC Press, Boca Raton, Florida.
Ziegler, R., Eckart, K., Schwarz, H. and Keller, R. 1985. Amino acid sequence of *Manduca sexta* adipokinetic hormone elucidated by combined fast atom bombardment (FAB)/tandem mass spectrometry. Biochem. Biophys. Res. Commun. 133: 337-342.

STRUCTURE, EXPRESSION, AND EVOLUTION OF THE *D. melanogaster* DPKQDFMRF-AMIDE NEUROPEPTIDE GENE

P.H. Taghert, L.E. Schneider, and M.A. O'Brien

Department of Anatomy and Neurobiology
Washington University School of Medicine
660 South Euclid Avenue
St. Louis, MO 63110

INTRODUCTION

In recent years, there has been a tremendous increase in the amount of information detailing the structure and expression of biologically active neuropeptides. While we have exact knowledge of many neuropeptides, the promise of a better understanding of what they do, both as individuals and as a class, and of how their diversity improves neuronal function, remains largely unfulfilled. Because of its diminutive size, *Drosophila melanogaster* has not been a favored subject for physiological studies. The study of neuropeptide expression and function in *D. melanogaster* is highly attractive, however, because of the advanced genetics and molecular techniques that are available for studies of gene regulation and function *in vivo*. The functions of neuropeptides are often difficult to analyze because of their structural diversity and because of a lack of specific antagonists. However, in *D. melanogaster*, the ability to create gene mutations offers the possibility of examining animals that chronically lack specific neuropeptide gene expression. Furthermore, the ability to transform the *D. melanogaster* germ line via P elements (Rubin and Spradling, 1982) means that such expression could be re-introduced to the nervous system, and in this way, neuropeptide function may be defined by the rescue of mutant phenotypes. With these ideas in mind, we initiated experiments to describe a *D. melanogaster* neuropeptide gene, its organization and its expression, as starting points from which to undertake a genetic analysis.

Our studies focus on a gene that encodes a series of FMRFamide-like neuropeptides. FMRFamide was first isolated as a cardioactive tetrapeptide from clams (Price and Greenberg, 1977); FMRFamide and FMRFamide-like neuropeptides have since been discovered in a variety of animals including insects (Nambu et al., 1988), crustacea (Trimmer et al., 1987), and chordates (Yang et al., 1985). *D. melanogaster* pre-pro-FMRFamide (Nambu et al., 1988; Schneider and Taghert, 1988) is an excellent model for the study of neuropeptide expression and function because it conforms in many ways to features described for other neuropeptide precursors. It contains many related neuropeptides, and also has protein regions not obviously coding for neuropeptides. It is expressed by a small subset of neurons within the CNS, but these neurons are not obviously related by cell lineage, cell body position, or axonal projection. This chapter reviews our recent progress in the study of this *D. melanogaster* gene: we describe the structure of the gene, its evolutionarily conserved features, and the pattern of neurons that express it.

THE NEUROPEPTIDE PRECURSOR

Based on the sequence of a cDNA isolated from a *D. melanogaster* head library, the

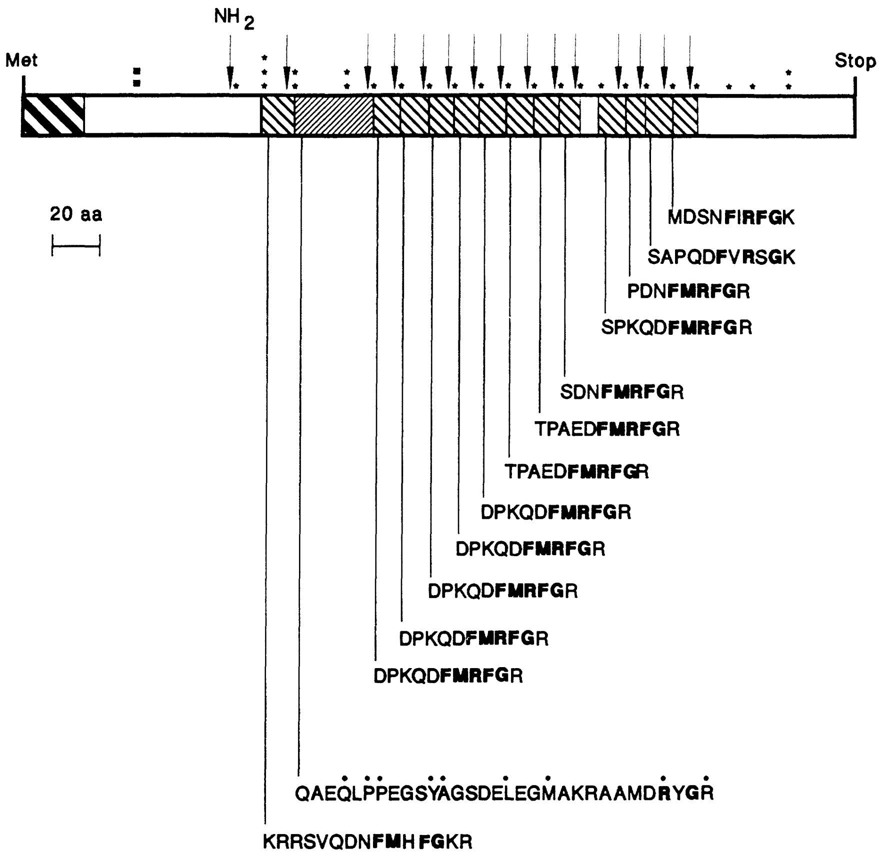


Figure 1. Schematic representation of the FMRFamide polyprotein precursor in *D. melanogaster*. The protein begins with a hydrophobic leader sequence (large diagonals). Deduced cleavage products (13 FMRFG-like peptides - medium diagonals- and a CRF-like peptide- small diagonals) are indicated. Gly residues (arrows) donate amide moieties for peptide amidation (NH_2) and basic residues (stars indicate number) are likely sites for endoproteolytic cleavage. The deduced peptides are listed below with FMRFG sequences in boldface. Dots above the longest peptide sequence indicate residues with homology to CRF and/or related peptides. Reprinted with permission from Schneider and Taghert (1988).

structure of the insect pre-pro-FMRFamide was deduced (Nambu et al., 1988; Schneider and Taghert, 1988). This large (~45 kDa) protein contains numerous, potentially bioactive peptides that end in the sequence -FMRFamide. The large number of such peptides within a single precursor, their sequence diversity, and their differing representation (i.e. copy number) all suggest neuropeptide involvement in a host of complex physiological processes. As diagrammed in Figure 1, the 347 amino acid precursor beings with a largely hydrophobic leader region; this region includes sequences that resemble consensus elements that direct (Haeuptle et al., 1989) and that undergo (von Heijne, 1983) signal peptide cleavage. These sequence features allow us to predict that such cleavage occurs following residue #17 (Ala). There are thirteen potential FMRFamide-containing neuropeptides that may be cleaved from this polyprotein. Each is bounded by single, di- or tribasic residue (potential signals for endo-proteolytic cleavage) and each is extended by 3-5 amino acids in the N-terminal direction. The peptides do not have identical sequences, but neither is each unique. Rather, there are 8 unique species; 5 tandem copies of the peptide DPKQDFMRFG and 2 copies of TPAEDFMRFG are found. All other FMRFamide-containing peptides are present only

once. Nambu et al., (1988) purified and sequenced a *D. melanogaster* peptide with the sequence DPKQDFMRFamide. This result supports the hypothesis that the suspected post-translational processing steps necessary to liberate and amidate all the individual FMRF-amide-like peptides do occur *in vivo*.

Within the precursors, the 13 FMRFamide-containing peptides are separated into three groups by two spacer regions. The first spacer is long and may itself contain biologically active species, as it demonstrates homology to the family of corticotropin-releasing factor-like peptides (Vale et al., 1983). This feature was previously noted in the case of the *Aplysia* pro-FMRFamide precursor (Taussig and Scheller, 1986). Other potential cleavage sites are present in the *D. melanogaster* precursor; the degree to which they are utilized and the functions of the precursor regions that do not contain FMRFamide-like peptides are uncertain.

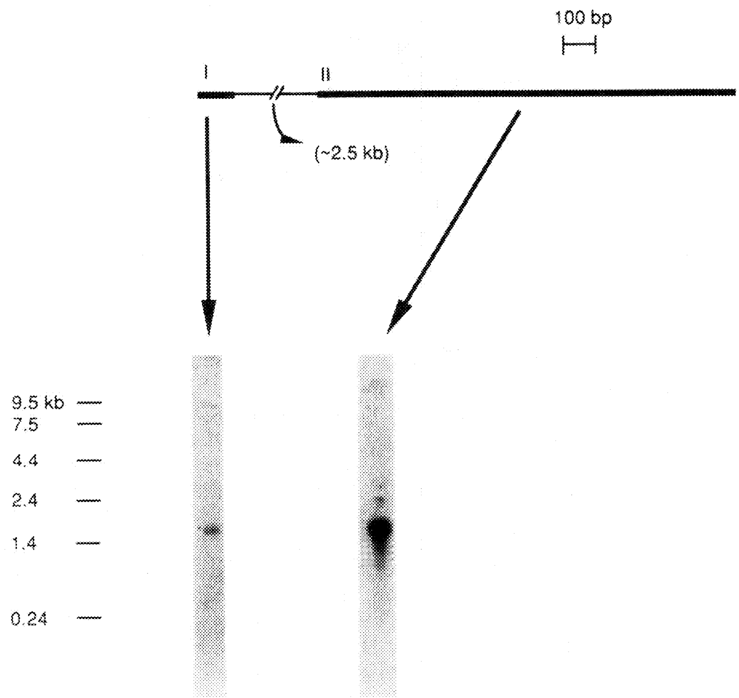


Figure 2. Northern blot analysis of neuropeptide gene expression in 1st instar larvae of *D. melanogaster*. 20 μg samples of poly A^+ RNA were probed independently (arrows) with genomic DNA regions containing the two exons (marked above by Roman numerals) of the *D. melanogaster* FMRFamide gene. RNA size markers are indicated to the left. Both probes hybridize to a prominent ~1700 nt RNA species.

GENE ORGANIZATION AND RNA EXPRESSION

Using primer extensions, nuclease protection assays, and genomic DNA sequencing, we determined that the FMRFamide neuropeptide gene is organized into two exons that are separated by an intron of about 2.5 kb (Schneider and Taghert, in press). Exon I is 106 bp long and completely untranslated; exon II is 1322 bp long and contains a single long open reading frame that encodes a 347 amino acid protein. We examined the expression of the gene with Northern blot analysis of RNA from different post-embryonic stages. As shown in Fig. 2 for the larval stage, a single transcript of ~1.7 kb hybridizes to DNA probes that represent either the exon I or exon II region. There is no evidence at present to indicate that alternative splicing takes place. These (and other) experiments indicate that the 1.7 kb mRNA is the predominant (perhaps exclusive) transcript of the neuropeptide gene in all post-embryonic stages.

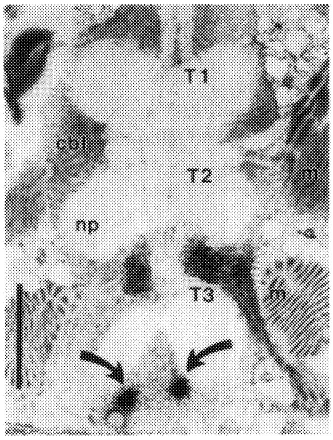


Figure 3. *In situ* hybridization of FMRFamide mRNA in a section of the adult *D. melanogaster* CNS. A 3' end-labeled oligonucleotide (a 41-mer, residues 802-843 of exon II) was used to probe the distribution of neuropeptide gene transcripts. Large hybridization signals (arrows, two are present in this section) correspond to positions of the T3v neurons, two pairs of FMRFamide-immunoreactive neurons of the 3rd thoracic neuromere. The tissue section was counterstained with haematoxylin/eosin; the neuropil (np) is unstained and the cell body layer (cbl) is stained. Further abbreviations: T1, T2 and T3, first, second and third thoracic neuromeres; m, muscles adjacent to CNS. Scale = 100 μ m.

MAPPING NEUROPEPTIDE GENE TRANSCRIPTS WITHIN THE CNS

With knowledge of the neuropeptide gene sequence, we developed oligonucleotide probes to visualize the number and patterning of CNS neurons that express specific gene transcripts. We surveyed all developmental stages to ascertain at which stages transcripts were detected and the degree to which the pattern changed during metamorphosis (Schneider, O'Brien, and Taghert, unpublished). A 24-mer probe (specific for Exon I) and a 41-mer probe (specific for exon II) were 3' end-labeled and hybridized to frozen sections of whole *D. melanogaster*; an example of hybridization to the adult CNS is shown in Fig. 3. Although it lacks precise cellular resolution, this method does provide a background low enough to detect even faint signals. We detected two classes of signals. The first class included neurons that contained high steady-state levels of the neuropeptide mRNA (Fig. 3). The second class (data not shown) gave very much reduced (though reproducible) signals. Interestingly, hybridization signals from neurons in the brain (i.e. the supra-esopha-geal ganglion) were at all times of the second, weaker class.

From these studies, we made five observations: (i) the pattern of hybridization signals corresponded very well to the majority of the ~50 neurons that are immuno-labeled by antisera to authentic FMRFamide (c.f., Fig. 5); (ii) neuropeptide gene transcripts were first detectable in late (17-21 hr, 22°C) embryos; from the outset, the pattern of 28 discrete hybridizing elements appeared constant through all larval stages; (iii) signals were not diminished during adult development (four time points examined), and they continued into adult stages (pharate, 6 hr, 3 and 10 day time points examined); (iv) the majority of adult signals was due to persistent, larval neurons; and (v) a small number of adult-specific signals were detected in both the brain and ventral nervous system of the CNS. We concluded that, first, a large proportion of the FMRFamide-like immunoreactivity in *D. melanogaster* is due to expression of the DPKQDFMRFamide gene. Second, the data indicated that specific gene expression continues throughout the animal's lifetime, including adult development, when the insect's behavioral repertoire is minimally engaged. Finally, despite the fact that approximately ninety percent of the neurons of the adult *D. melanogaster* CNS are created post-embryonically (Truman, and Bate, 1988), it appeared that the majority of FMRFamide neuropeptide functions are not stage-specific. This conclusion is drawn on the fact that most

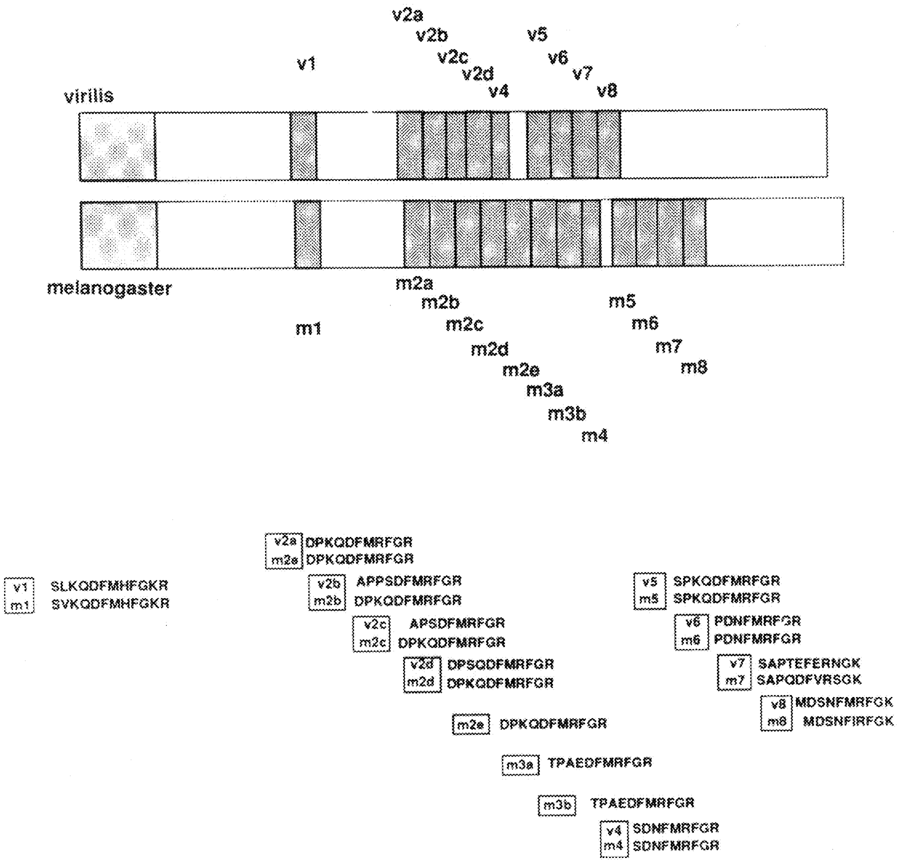


Figure 4. The FMRFamide precursors of *D. virilis* and *D. melanogaster*. Top: Schematic diagrams of the proteins placed with the initiator methionines in register. The signal sequences are indicated by lightly-filled boxes at the extreme left hand ends. Dark boxes within each protein indicate positions of FMRFamide-like peptides, v1-8 and m1-8 respectively. Bottom: Direct comparison of the peptides according to the registration of the proteins indicated above. The peptide names are boxed.

of the neurons that displayed hybridization in the adult were persistent larval neurons; hence, FMRFamide neuropeptide functions in the adult are likely to be very similar to those in larval stages. The use of antisera to specific peptide epitopes of the neuropeptide precursor (Schneider and Taghert, work in progress) is providing precise cellular resolution to complement these maps of neuropeptide mRNA within the *D. melanogaster* CNS.

EVOLUTION OF THE *Drosophila melanogaster* DPKQDFMRFamide GENE

In order to identify functionally important regions of the neuropeptide gene, we isolated and characterized a homologous gene from *Drosophila virilis* (Taghert and Schneider, in press). This species is estimated to have diverged from *D. melanogaster* between 40 and 60 Myr ago (Throckmorton, 1975). This comparison led to the following conclusions. (i) The genes are similarly divided into two exons; in each species, exon I is completely untranslated. (ii) Exon I is poorly conserved between the species, with sequence similarities present only at the start site. (iii) The deduced precursors have very similar sizes and organizations (Fig. 4). (iv) The *D. virilis* precursor contains 10 FMRFamide-like neuro-peptides, com-

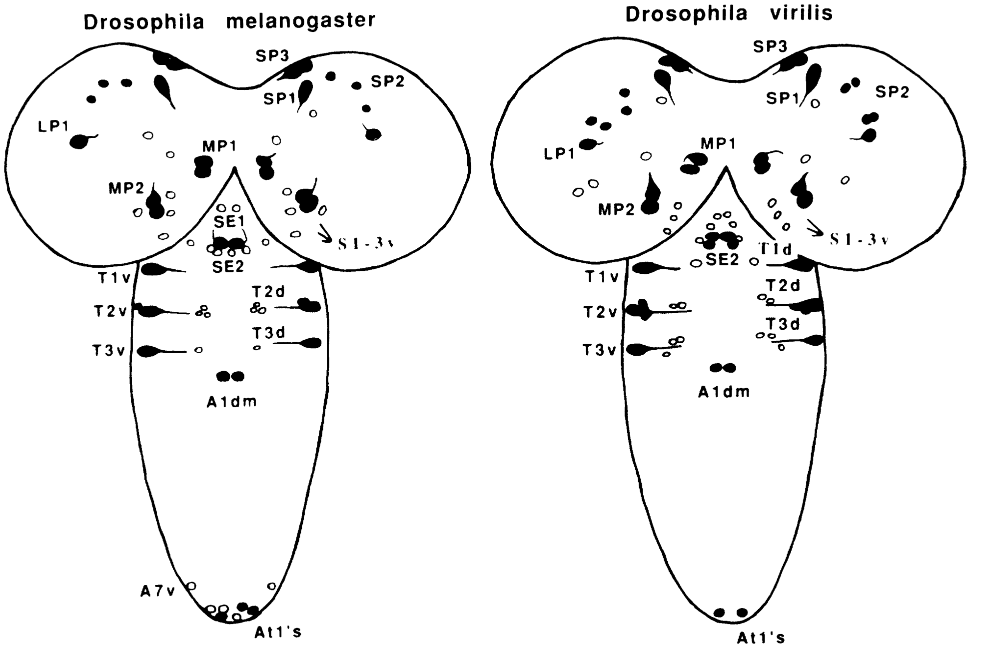


Figure 5. Schematic diagrams that indicate the similarity in number and position of FMRFamide-immunoreactive cell bodies in the central nervous systems of *D. melanogaster* and *D. virilis*. Filled symbols represent strongly-staining neurons and open symbols represent lightly-staining neurons. Abbreviations as follows: MP, LP, and SP, medial, lateral and superior protocerebrum; SE, subesophageal; S1-3 and T1-3, neurons of subesophageal and thoracic segments 1-3 respectively; A1-7, neurons of abdominal segment 1-7 respectively. Small case d and v indicate dorsal versus ventral cell body positions; small case m indicates a medial cell body position.

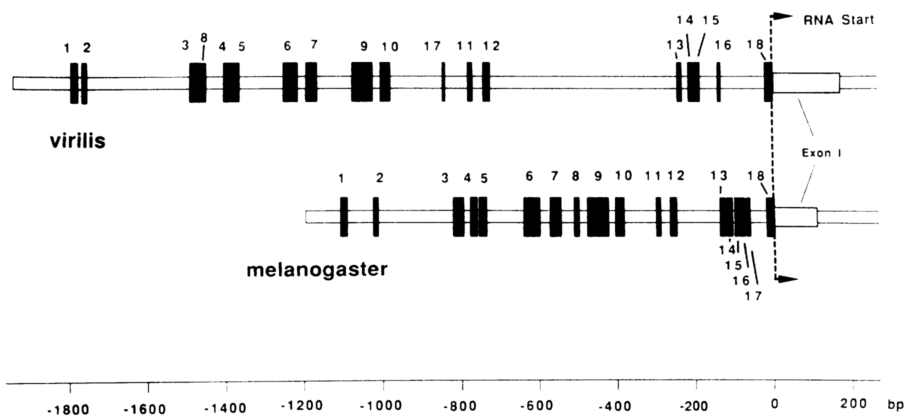


Figure 6. A map of conserved regions that are 5' to the RNA start site of the FMRFamide gene in *D. melanogaster* and *D. virilis*. These regions (black boxes) are 9-52 bp in length and in *D. melanogaster* are numbered #1-18 consecutively, beginning with the region most distal to the start site. Region #18 begins with a canonical TATA box sequence and ends just after the start site. Note that exon I is larger in *virilis* and does not show significant sequence similarity except at the start site.

pared to thirteen in the case of *D. melanogaster*. (v) All FMRFamide-like peptides are extended in the N-terminal direction; some extensions are conserved between the two species, while others are divergent. (vi) Comparing precursor regions that do not code for FMRFamide-like peptides, the N-terminal half displays considerable sequence conservation, while the C-terminal half does not. Immediately following the final FMRFamide-like peptide, the protein sequences diverge.

The precursors have approximately a 59% overall level of amino acid identity. The highest degrees of conservation, in addition to that within the hydrophobic leader sequence, are found in the 3 regions encoding FMRFamide-like peptides. With the exception of two conservative changes, all single, di- and tri-basic amino acid residues, which are presumed to be cleaved to liberate the FMRFamide-like peptides, are identical between the two species. Although the *D. virilis* precursor contains three fewer FMRFamide-like peptides, it is nevertheless of the same approximate size as that of *D. melanogaster*, 339 versus 347 amino acids (Fig. 4). The compensated length occurs in *D. virilis* through the extension of the C-terminal region of the precursor, and therefore is composed entirely of divergent squences. This observation suggests an evolutionary pressure to preserve precursor size and may relate to the optimization of precursor processing and/or packaging within secretory granules.

The comparison of precursor structure between the two *D. melanogaster* species (Fig. 4) indicates that while the basic organization of peptides within the precursor was conserved, there is only a limited degree of conservation in peptide sequences and substantial variability in peptide copy number. In both precursors, three non-contiguous groups of FMRFamide-like peptides are found: these three groups include peptide 1, peptides 2-4, and peptides 5-8 respectively. Based on the degree of sequence conservation (Fig. 4), and assuming that homologous neuropeptides perform similar functions in both *D. melanogaster* species, we suggest that these three groups may represent functionally distinct sets of neuropeptides. For example, peptides in groups 1 (m1 and v1) and 3 (m5-8 and v5-8) are highly conserved, whereas peptides in group 2 (m and v2/3) show considerable sequence divergence. The variability in copy number of the second group may indicate that absolute numbers of such peptides are not critical for proper function. We suspect that, while multiple copies of a single peptide sequence are advantageous, only a minimum number may be needed. Extra copies may provide latitude for new sequences to arise that are free from strong negative selection (compare v2 to m2 sequences). Variations in the extension sequences of the FMRFamide-like peptides may also result in differences in the degradation rates following release: N-terminal extended forms of FMRFamide in *Helix* are known to have longer half-lives (Price, 1986).

THE NEUROPEPTIDE GENE PROMOTER

We have also begun to examine the developmental mechanisms by which neuropeptide gene expression is restricted to a stereotyped subset of neurons within the CNS. While it may be the case that all neurons express this gene at a very low level, *in situ* hybridization studies have indicated that a particular subset contained very high steady-state levels of the neuropeptide mRNA (Fig. 3). It is therefore reasonable to suppose that, as is the case for other neuropeptide genes and transmitter biosynthetic enzyme genes (Rosenfeld et al., 1984; Scheller et al., 1984; Beall and Hirsch, 1987), differential gene expression is the primary basis for the establishment of this cell-specific neuronal phenotype. The map of FMRFamide-immunoreactive neurons is nearly indistinguishable between *D. melanogaster* and *D. virilis* (Fig. 5); only slight differences in the number of immunoreactive neurons per cell group were evident (e.g., the Tv cell group). We concluded therefore that mechanisms directing expression to this conserved subset of neurons are likely to be equally conserved. Previous studies have demonstrated the utility of evolutionary comparisons between these two species (Blackman and Meselson, 1986; Treier et al., 1989; Johnson et al., 1989). We therefore compared DNA sequences upstream of the RNA start site looking for conserved regions as putative candidates for regulatory elements.

A schematic diagram of the regions surveyed is shown in Fig. 6. Approximately 1.2 kb of D. *melanogaster* DNA and 1.8 kb of D. *virilis* DNA are indicated: although these sequences are highly divergent for the most part, we found 18 regions (9-52 bp long) that showed significant (>90%) sequence conservation. The last (#18) comprises a 32 bp sequence that contains a match with the canonical TATA box and that ends with the RNA

start site. While these results do not define the limits of the promoter region, they suggest that it is likely to be a distributed function, not limited to the immediate vicinity of the gene. Current efforts are directed toward understanding the extent to which these putative elements are involved in neuropeptide gene regulation.

PROSPECTS FOR A GENETIC ANALYSIS OF NEUROPEPTIDE EXPRESSION

We have described the structure, expression, and evolution of a neuropeptide gene in *D. melanogaster*. In doing so, our goal has been to formulate a basis from which to use the advanced genetics and molecular techniques that are available for this animal to address questions regarding neuropeptide gene regulation and function. The FMRFamide gene is located in band 46C of the 2nd chromosome (Schneider and Taghert, 1988). A small number of deficiencies have been mapped to this region (Nüsslein-Volhard et al., 1984); unfortunately, they do not cover the neuropeptide gene. In order to establish stocks of animals that are mutant in the expression of this gene, we have created new deficiencies in the 46C region by using X-rays to revert closely-positioned P elements that bear selectable markers (O'Brien and Taghert, unpublished). Success in this initial aspect of mutagenesis should permit future attempts to create point mutations in the gene for a more precise genetic dissection. Our goal in these experiments is an *in vivo* examination of the physiological and developmental consequences of manipulating neuropeptide gene structure and dosage. Against a mutant (null) background, structurally altered FMRFamide genes can be stably integrated into the germ line so as to test hypotheses concerning the functions, copy number, and linear order of multiple neuropeptides within a single polyprotein precursor. Similar methods would also be useful to complement parallel studies of the gene promoter and the rules by which precise, cell-specific expression of a neuropeptide gene is elaborated during development.

ACKNOWLEDGMENTS

We thank Jay Hirsch for communicating unpublished results. We are grateful to acknowledge support for this work by an NIH Post-Doctoral Fellowship to MO'B and by grants to PHT from the NIH (NS21749) and the Washington University-Markey Center in the Molecular Biology of Human Disease.

REFERENCES

Beall, C.J. and Hirsch, J. 1987. Regulatiion of the *D. melanogaster* Dopa decarboxylase gene in neuronal and non-neuronal cells. Genes and Devel. 1: 510-520.

Blackman, R.K. and Meselson, M. 1986. Inter-specific nucleotide sequence comparisons used to identify regulatory and structural features of the *D. melanogaster* hsp82 gene. J. Mol. Biol. 188: 499-515.

Haeuptle, M.-T., Flint, N., Gough, N.M. and Dobberstein, B. 1989. A tripartite structure of the signals that determine protein insertion into the endoplasmic reticulum membrane. J. Cell. Biol. 108: 1227-1236.

Johnson, W.A., McCormick, C.A., Bray, S.J. and Hirsch, J. 1989. A neuron-specific enhancer of the *Drosophila melanogaster* dopa decarboxylase gene. Genes and Develop.. in press.

Nambu, J.R., Murphy-Erdosh, C., Andrews, P.C., Feistner, G. and Scheller, R.H. 1988. Isolation and characterization of a *Drosophila melanogaster* neuropeptide gene. Neuron 1: 55-61.

Nüsslein-Vollhard, C., Weischaus, E. and Kluding, H. 1984. Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster*. I. Zygotic loci on the second chromosome. Roux's Arch. Devel. Biol. 193: 267-282.

Price, D.A. 1986. Evolution of a molluscan cardioregulatory neuropeptide. Amer. Zool. 26: 1007-1017.

Price, D.A., Greenburg, M.J. 1977. Structure of a molluscan cardioacceleratory peptide. Science 197: 670-671.

Rosenfeld, M.G., Amara, S.G. and Evans, R.M. 1984. Alternative RNA processing: determining neuronal phenotype. Science 225: 1315-1320.
Rubin, G.M. and Spradling, A. 1982. Genetic transformation of *Drosophila melanogaster* with transposable element vectors. Science 218: 348-353.
Scheller, R.H., Khaldany, R.R., Kreiner, T., Mahon, A.C., Nambu, J.R., Schaeffer, M. and Taussig, R. 1984. Neuropeptides: Mediators of behavior in *Aplysia*. Science 225: 1300-1308.
Schneider, L.E., and Taghert, P.H. 1988. Isolation and characterization of a *Drosophila melanogaster* gene encoding multiple neuropeptides related to FMRFamide (Phe-Met-Arg-Phe-NH_2). Proc. Natl. Acad. Sci. USA 85: 1993-1997.
Schneider, L.E., and Taghert, P.H. 1990. Organization and expression of the *Drosophila* Phe-Met-Arg-Phe-amide neuropeptide gene. J. Biol. Chem. In press.
Taghert, P. H. and Schneider, L. E. Interspecific comparison of a Drosophila neuropeptide gene encoding FMRF-amide-related peptide. J. Neurosci. in press.
Taussig, R. and Scheller, R.H. 1986. The *Aplysia* FMRFamide gene encodes sequences related to mammalian brain peptides. DNA 5: 453-461.
Throckmorton, L.H. 1975. pp 421-469 in: "Handbook of Genetics." Vol. 3. King, R.C,. ed. Plenum Press, New York.
Treier, M., Pfeifle, C. and Tautz, D. 1989. Comparison of the gap segmentation gene hunchback between *Drosophila melanogaster* and *D. virilis* reveals novel modes of evolutionary change. EMBO J. 8: 1517-1525.
Trimmer, B.A., Kobierski, L.A. and Kravitz, E.A. 1987. Purification and characterization of FMRFamide-like immunoreactive substances from the lobster nervous system: Isolation and sequence analysis of two closely related peptides. J. Comp. Neurol. 266: 16-26.
Truman, J.W. and Bate, C.M. 1989. Spatial and temporal patterns of neurogenesis in the central nervous system of *Drosophila melanogaster*. Devel. Biol. 125: 145-157.
Vale, W.W., Rivier, C., Spiess, J. and Rivier, J. 1983. pp. 67-73 in: "Brain Peptides." Dreiger, D., Brownstein, M.J. and Martin, J.B., eds. Wiley, New York.
von Heijne, G. 1983. Patterns of amino acids near signal-sequence cleavage sites. EMBO J. 5: 3021-3027.
Yang, H.-Y.T., Fratta, W., Majane, E.A. and Costa, E. 1985. Isolation, sequencing, synthesis and pharmacological characterization of two rat brain neuropeptides that modulate the actions of morphine. Proc. Nat. Acad. Sci. USA 82: 7757-7761.

MOLECULAR APPROACHES TO THE PRODUCTION AND ACTION OF ECLOSION HORMONE

James W. Truman, Frank M. Horodyski, David B. Morton
and Lynn M. Riddiford

Department of Zoology
University of Washington
Seattle, WA 98195 U.S.A.

INTRODUCTION

Ecdysis involves more than just the shedding of an old cuticle. The ecdysis of the adult, for example, is the time when the new imago first confronts the external world, and at this time many behavioral and physiological systems first become activated. Studies on the hormonal regulation of this event began 20 years ago with the report that a transplanted brain could control the timing of ecdysis of a debrained host (Truman and Riddiford, 1970). A factor that induces precocious ecdysis was subsequently extracted from the brain and termed the eclosion hormone (EH; Truman, 1971). The sole known function of this hormone is to coordinate the events that occur at ecdysis. It acts directly on the central nervous system to release the appropriate ecdysial behaviors (e.g., Truman, 1978) but also has ecdysis-related effects on non-neural targets such as epidermis (Reynolds, 1977) and muscle (Schwartz and Truman, 1982).

The molecular approach to the study of the EH system has been two-pronged. One direction has involved the hormone itself: its structure, the gene that codes for it, and the way in which its expression is regulated. The second direction relates to the pathway that mediates the response to EH. This pathway includes the EH receptors as well as the other proteins that are downstream in the cascade that is started by EH and that ends with the performance of the appropriate behaviors. The last few years have been marked by some important advances in the study of EH and its action. This paper will briefly review these advances and their implications for future work in this area.

THE SEQUENCE OF ECLOSION HORMONE AND ITS GENE

The eclosion hormone (EH) of *Manduca sexta* was extracted from trimmed heads or isolated corpora cardiaca-corpora allata (CC-CA) complexes, purified by reverse-phase, high-performance liquid chromatography, and subjected to microsequencing techniques (Marti et al., 1987; Kataoka et al., 1987). The hormone was found to be an unblocked, acidic peptide composed of 62 amino acids, including 6 cysteine residues. The cysteins form disulfide bridges that are essential for EH activity, as their reduction abolishes biological activity (Terzi et al., 1988). The structure of EH from *Bombyx mori* was also reported at the same time (Kono et al., 1987), and the peptides of the 2 species share 80% of their residues in common including the cysteines. Because they have no similarity to any other known peptides, the EHs appear to constitute a novel set of neuropeptides.

Molecular Insect Science, Edited by
H. H. Hagedorn *et al.*, Plenum Press, New York, 1990

The cloning of the eclosion hormone gene from Manduca

The determination of the primary structure of EH provided an avenue for the isolation of the gene that codes for it. (Horodyski et al., 1989). A unique oligonucleotide probe was designed based on the N-terminal 24 amino acids of *M. sexta* EH. The probe was used to screen a *M. sexta* genomic library, and hybridized to 8 clones which represented 3 genomic regions. A restriction fragment from one of the clones was sequenced and found to contain most of the EH sequence. This restriction fragment was then used to isolate the corresponding cDNA clone from a library prepared from brain RNA.

The organization of the EH gene is summarized in Fig. 1 (Horodyski et al., 1989). The gene extends over 7.8 kb of DNA and is organized into 3 exons. The first exon contains only 5' untranslated sequence and is separated from the second exon by about 3 kb. The second exon comprises 102 bp and codes for the hydrophobic signal peptide and the first 4 residues of EH. The remainder of the EH sequence is found on the third exon, situated 4.1 kb downstream. The mature mRNA from the gene is 0.8 kb in size and codes for an 88 amino acid preEH consisting of a 26 amino acid signal sequence followed by a single copy of EH. Northern blot analysis of RNA extracted from various regions of the nervous system showed detectable message only in the brain. Transcripts were found in the brains of larvae and diapausing pupae, and throughout adult development.

The search for eclosion hormones from other insects

The determination of the complete sequence of *M. sexta* EH required 50-75 kg of insects for the tissue needed for hormone extraction (Marti et al., 1987; Kataoka et al., 1987). This reflects the fact that EH is present in very small amounts (about 3.4 ng/insect). In the face of low levels of peptide that are likely to be present, the prospects of using conventional means for isolating and sequencing EHs from other insects outside of the large Lepidoptera are not encouraging. EH is broadly cross-reactive amongst the insects (Truman et al., 1981), however, suggesting considerable conservation in structure. Consequently, it seemed reasonable to use the EH gene from *M. sexta* to isolate EHs from other species.

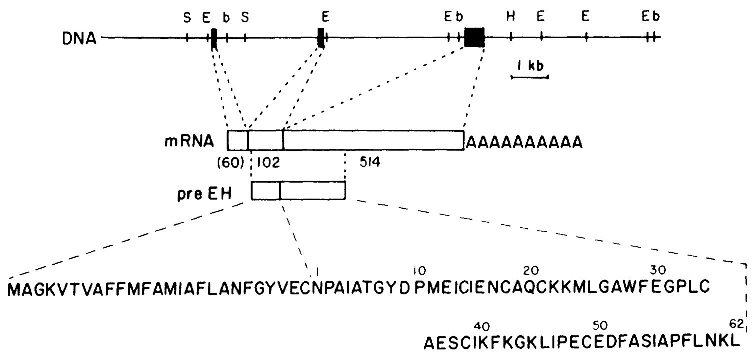


Figure 1. Organization of the *Manduca sexta* EH gene. The top line shows the restriction map of a portion of the genomic clone indicating the location of the 3 exons of the EH gene. Subsequent lines show the relationship of the mature mRNA to the EH precursor (PreEH). For the mRNA, the numbers refer to the number of nucleotides in each exon. The amino acid sequence of PreEH is given below; it includes the signal sequence (left) followed by residues 1 to 62 of EH. The restriction enzymes are: b, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; S, *Sau*3A. (Modified from Horodyski et al., 1989).

A DNA fragment containing the *M. sexta* EH coding region was used to screen a *Drosophila melanogaster* genomic library. A portion of the cross-hybridizing clone was sequenced and found to contain an exon that coded for the C-terminus of the *D. meloanogaster* EH. This region of the fly EH shares about 70% of its amino acids in common with those from *M. sexta*, including the positions of the cysteines (Horodyski, Riddiford & Truman, unpublished). A probe derived from this region detects a 0.8 kb

transcript in *D. melanogaster* pupae. The comparative studies on *D. melanogaster* and other species are still in their early stages, but they will provide some insight into how the structure of EH has diversified during insect evolution. These studies should also identify conserved portions of the molecule, thereby indicating regions that are most important for biological activity.

The cloning of the EH gene also provides an avenue for generating synthetic EH. The large size of the peptide as well as the need to form the appropriate disulfide bridges pose some problems for peptide synthesis by conventional techniques. With the gene in hand, yeast (Ammerer, 1983) or baculovirus (Maeda, 1989) expression systems may provide a means for generating synthetic peptide that can then be used for physiological studies and for receptor isolation.

THE ECLOSION HORMONE NEURONS

Based on physiological criteria and immunological staining, Copenhaver and Truman (1986) identified a set of neurons in the lateral neurosecretory cell (NSC) group that project to the CC-CA complex and release EH at adult ecdysis. These Group Ia cells, however, were clearly not the only cells involved in EH secretion. During larval and pupal ecdysis, the CC-CA complex is devoid of EH activity, and EH release occurs from the ventral nervous system (Truman et al., 1981). Since the Group Ia cells do not project into the ventral CNS, another set of cells had to be making EH during these earlier stages.

Quantification of the levels of EH activity in the prepupal nervous system showed that EH was distributed throughout the CNS but found in only one peripheral site, the proctodeal nerves (Truman and Copenhaver, 1989). Indeed, the level of EH activity stored in these nerves was greater than that found in the rest of the nervous system combined. The amount of peptide found in the proctodeal nerves fluctuated cyclically, gradually accumulating during the intermolt period and through the early phases of the molt and then abruptly dropping by about 90% at ecdysis. This pattern of EH accumulation and depletion is consistent with the conclusion that the proctodeal nerve is the site of EH release for larval and pupal ecdyses.

A series of lesion experiments were used to identify the site of EH production (Truman and Copenhaver, 1989). Irrespective of where lesions were placed along the nerve cord, EH activity accumulated rostral to the lesion and disappeared caudal to it. Importantly, the removal of one side of the brain resulted in the loss of activity in the proctodeal nerve on the lesioned side but normal activity on the intact side. Thus, there must be brain neurons whose axons project the length of the CNS and into the proctodeal nerve.

Backfills of the proctodeal nerves with cobalt ions, as well as immunostaining with an antibody against EH, confirmed the existence of such cells. The brains of larval and pupal *M. sexta* contain two pairs of neurons whose cell bodies are situated in the ventromedial region and whose axons extend ipsilaterally in a dorsomedial tract through the ventral CNS and into the proctodeal nerves, where they form beaded varicosities. *In situ* hybridization using a radiolabeled probe directed against the EH mRNA showed strong hybridization to these cells (Horodyski et al., 1989), indicating that they contain EH message as well as EH immunoreactivity.

These neurons, the ventromedial EH neurons (VM cells), constitute a novel neurosecretory pathway--the brain-proctodeal system (Truman and Copenhaver, 1989). They also pose some interesting questions relating to the evolution and functioning of the EH system. One question is the relationship of these cells to the Group Ia NSCs described by Copenhaver and Truman, (1986). At larval and pupal ecdyses, the VM cells appear to be the sole source of EH activity in the CNS. The Group Ia cells are present in these immature stages, but no EH activity is present in the CC-CA complex where their axons terminate. Moreover, based on in situ hybridization, significant levels of EH mRNA are found in the larval brain only in the VM cells (Horodyski et al., 1989). The EH activity found in the ventral CNS appears to be due to peptide in transit down the axons of the VM cells. This conclusion is supported by a number of observations: the axons of the VM cells are the only immunopositive structures in the ventral CNS, lesions that sever the axons consistently result in the loss of EH activity in the CNS distal to the lesions (Truman and Copenhaver, 1989), and Northern blot analysis of RNA extracted from various regions of the CNS detected EH transcripts only in the brain (Horodyski et al., 1989). These findings, of course, cannot exclude the possibility that a few cells in the ventral ganglia make small amounts of

EH that are below our limits of detection. Nevertheless, the VM cells clearly account for the majority, if not all, of the EH in the nervous systems of larval and pupal *M. sexta*.

EH first appears in the CC-CA complex during the transition from pupa to adult (Truman et al., 1981). The lateral cells apparently begin to produce EH during this time and are responsible for at least part of the activity found in the CC-CA prior to adult ecdysis. Interestingly, in the adult stage, the VM cells also project to the CC-CA. Immunostaining of the brains from pharate adults shows that the adult form of the cell has axon collaterals that extend to the CC-CA complex. Thus, these cells sprout a second axon during metamorphosis so that at adult ecdysis they release hormone from both anterior and posterior release sites. The relative contribution of the VM cells versus the Group Ia lateral cells to the control of adult ecdysis remains to be determined.

The VM cells are not unique to Manduca sexta

Immunocytochemical studies on both the fruitfly, *D. melanogaster,* and the house cricket, *Acheta domesticus*, reveal brain neurons that have a similar anatomy and that stain with the anti-EH antiserum (Truman, unpublished). *D. melanogaster* possesses only one pair of VM neurons rather than the 2 pairs seen in *M. sexta*. In fly larvae, these cells are located in the ventromedial region of the brain, and their axons project ipsilaterally through the length of the ventral CNS. In the adult, the cells have an axon collateral that extends towards the CC-CA, but we have not yet been able to follow it in its entirety. The cricket, by contrast, possesses 2 pairs of large, immunopositive cells in the ventromedial region of its brain. The staining in the brain has not yet been adequate to reveal a process to the CC-CA, but the latter contains a large store of EH. Immunopositive axons leave the brain via the circumesophageal connectives and travel through the ventral CNS to the terminal nerve, where they exit via a side branch leading to the proctodeum. Interestingly, in the cricket these axons elaborate a spray of processes in the rostral and caudal regions of each ganglion through which they run. The unique morphology of these cells in the cricket, moth, and fly suggests that they are indeed homologues and that cells of this morphology are probably present in most insect nervous systems. Moreover, these cells appear to have been associated with EH production through most of insect evolution.

An intriguing aspect of the VM cells in crickets is the prominent branching seen in each segmental ganglion. At this time, we do not know whether these are input or output sites, but the presence of EH in them would suggest the latter. If so, firing of the VM cells would result in local EH release in each ganglion as well as secretion into the peripheral circulation. This prospect of local release of EH raises questions as to the avenue by which EH normally triggers ecdysis. It is clear in moths that blood-borne EH can act directly on the CNS to evoke these behaviors (e.g., Truman, 1978), but is circulating hormone necessary for triggering the behavior or can locally released peptide suffice?

An experiment that examines this question involves the ecdysis of semi-dissected pharate pupae in which the hindgut and its attached proctodeal innervation were reflected into a separate bath. Since the proctodeal nerve is the only known site of EH release at this stage, these animals should have no circulating EH. These insects undergo ecdysis on schedule, and associated with this process is a massive release of EH into the bath surrounding the hindgut (R. Hewes and J. Truman, unpublished). While these experiments are still preliminary, they suggest that circulating EH is not required to trigger ecdysis behavior and that local release in the various ganglia is sufficient to initiate the behavioral program. Consequently, the circulating hormone may be directed at peripheral targets such as muscles (Schwartz and Truman, 1982) and epidermis (Reynolds, 1977), rather than CNS.

PHOSPHOPROTEINS AS THE SUBSTRATES FOR EH ACTION

Over the past few years, considerable information has been gathered relating to the mode of action of EH. The peptide acts on target tissues, such as the nervous system (Morton and Truman, 1985) and muscles (Schwartz and Truman, 1984), to cause a rapid elevation in the levels of cyclic 3'5'-guanosine monophosphate (cyclic GMP). In the CNS, the cyclic GMP rise, in turn, activates a cyclic GMP-dependent protein kinase, which then phosphorylates two membrane associated proteins, the EGPs (for EH and cyclic GMP regulated phosphoproteins; Morton and Truman, 1988a).

The EGPs are low-abundance proteins that are loosely associated with the membrane fraction of the CNS and have a molecular weight of 54 kDa. In cell-free extracts they serve as substrates for either the cyclic GMP-dependent kinase or the cyclic AMP-dependent kinase, but *in vivo* their phosphorylation appears to be accomplished only by the former enzyme (Morton and Truman, 1988a). Evidence that they are phosphorylated *in vivo* in response to EH exposure comes from "back-phosphorylation" experiments (Morton and Truman, 1988a). Under this paradigm, animals of the same developmental stage were treated with either EH or saline. After an appropriate incubation period, the nervous systems were homogenized and incubated with ^{32}P-ATP in the presence of 0.1 mM cyclic GMP. The nervous systems that had been exposed to EH showed no incorporation of label into the EGPs, while those exposed to just saline showed a normal pattern of incorporation. The lack of label in the former group is interpreted as the result of the EGPs being phosphorylated with unlabeled phosphate in response to EH exposure and, hence, being unavailable during the *in vitro* labeling. The EGPs were the only proteins that consistently showed a loss of labeling after challenge with EH. At this time the manner by which the phosphorylation of these proteins alters the properties of the target cells to cause the onset of ecdysis behavior is unknown.

The biochemical basis for the "windows" of EH sensitivity

An intriguing aspect of the EH system is that the CNS is responsive to EH only for short periods of time just prior to each ecdysis (Morton and Truman, 1986, 1988a). The basis of these "windows" of responsiveness is seen in the changes in the biochemical cascade that mediates EH action, and they have been examined in detail for pupal ecdysis. A fifth instar larva, about 4 days prior to pupal ecdysis, shows no response to injection of EH. At this stage, the biochemical cascade that mediates EH action lacks at least two necessary components. Treatment with peptide at this time does not elevate the levels of cyclic GMP in the CNS (Morton and Truman, 1985). Thus, there appears to be a block between EH reception and the stimulation of its second messenger. Levels of guanylate cyclase are normal at this time, so the block is likely to be at the receptor or at the transduction step between receptor and cyclase. In addition to this block at the hormone reception/ tranduction end of the cascade, there is a second block at the mediational end--the EGPs are absent (at least as measured by their ability to be phosphorylated in an *in vitro* system).

The various components of the EH response system are then assembled during the larval-pupal transformation. The reception/transduction part of the system first becomes functional by about 24 hr before ecdysis. At this time injections with EH result in a normal cyclic GMP increase, but, importantly, there is no motor response that follows (Morton and Truman, 1985). The EGPs then appear about 8 hr before ecdysis and it is only at this time that EH evokes a complete neural response (Morton and Truman, 1988a). At this time the appearance of the EGPs can be followed only by their ability to incorporate labeled phosphate. Thus, the proteins themselves could be absent, or their ability to incorporate phosphate could be blocked. Experiments with cycloheximide show that protein synthesis is necessary just prior to the appearance of the EGPs (D. Morton and J.Truman, unpublished). Consequently, our working hypothesis is that they are synthesized *de novo* at about 8 hr before ecdysis. The trigger for their appearance is the decline in ecdysteroid levels that occurs at the end of the instar (Morton and Truman, 1988b).

The EGPs appear only transiently in the CNS. They are always present when the insect is responsive to EH, irrespective of whether it is a larval, pupal or adult ecdysis. Thus, their presence is associated not with a particular life stage but rather with the nervous system's being in a particular physiological state, ie. competent to respond to EH. During the larval and pupal molts, the EGPs appear to be the limiting factor in determining whether or not the CNS can respond to EH. During the adult transformation, however, the EGPs are found a number of days before responsiveness is established. Consequently, at this stage there appears to be yet another factor that is important for the establishment of full EH responsiveness. The nature of this additional factor is unknown.

Changes in phosphoproteins and the regulation of CNS function

A tissue's response to a hormone is commonly regulated by up or down regulation of receptors (Klein et al., 1989). Presumably, in intermolt larval *M. sexta*, a block at the re-

ceptor level is responsible for the inability of EH to evoke an increase in cyclic GMP. Intriguingly, however, a second block is present at the level of the phosphoprotein substrates. What is the reason for having two blocks? One hypothesis relates to the need of the CNS to cope with a large number of signaling molecules with only a few second messenger systems. The target cells that respond to EH have not yet been identified, but at least some of the cells involved are probably multifunctional neurons that participate in a number of behaviors. Accordingly, such cells would be tuned for EH-related activities during only about 5% of the life of the insect. If the EGPs were present continuously, then these neurons would be precluded from using cyclic GMP in conjunction with any other receptor/ligand system because the ligand would cause EH-like responses through the phosphorylation of the EGPs. Removal of the EH substrates as well as the receptors, however, leaves the cyclic GMP/kinase system free to work on other substrates to evoke a different suite of cellular responses. Consistent with this hypothesis is the finding that the levels of guanylate cyclase and the cyclic GMP-dependent protein kinase do not seem to fluctuate during the larval-pupal transition (Morton and Truman, 1988a).

The foregoing scenario is speculation at this point, but there is one experimental observation that is in line with such a hypothesis. The EH system is one of the first examples where alterations in the levels of phosphoprotein substrates regulate hormone responsiveness. Is this an isolated example? We examined the spectrum of phosphoproteins that are present in the CNS and are phosphorylated in a cyclic-nucleotide-dependent fashion at various times during the last larval stage and the larval-pupal transition (D. Morton and J. Truman, unpublished). There are at least 5 proteins besides the EGPs that change dramatically in their availability during this period. Each shows its own unique pattern of appearance and disappearance, one of which is exactly complementary to the pattern exhibited by the EGPs. Neither the function of these proteins, nor the nature of the ligands that induce their phosphorylation is known. Nevertheless, the existence of these proteins suggests that shifts in the spectrum of phosphoprotein substrates may be important for shifting the CNS from one behavioral state to another.

ACKNOWLEDGMENTS

Research was funded by grants from NSF (DCB-8615627 and BNS 8719611) and NIH (NS 25715).

REFERENCES

Ammerer G. 1983. Expression of genes in yeast using the ADC1 Promoter. Methods Enzymol. 101: 192-201.

Copenhaver P.F. and Truman J.W. 1986. Identification of the cerebral neurosecretory cells that produce eclosion hormone in the moth *Manduca sexta*. J. Neurosci. 6: 1738-1747.

Horodyski F.M., Riddiford L.M. and Truman J.W. 1989. Isolation and expression of the eclosion hormone gene from the tobacco hornworm, *Manduca sexta*. Proc. Natl. Acad. Sci. U.S.A. 86: 8123-8127.

Kataoka H., Troestschler R.G., Kramer S.J., Cesarin B.J. and Schooley D.A. 1987. Isolation and primary structure of the eclosion hormone from the tobacco hornworm, *Manduca sexta*. Biochem. Biophys. Res. Commun. 146: 746-750.

Klein, W.L., Sullivan, J., Skorupa, A., and Aguila, J.S. 1989. Plasticity of neuronal receptors. FASEB J. 3: 2132-2140.

Kono T., Nagasawa H., Isogai A., Fugo H., and Suzuki A. 1987. Amino acid sequences of the eclosion hormone of the silkworm, *Bombyx mori*. Agric. Biol. Chem. 51: 2307-2308.

Maeda, S. 1989. Expression of foreign genes in insects using baculovirus vectors. Ann. Rev. Entomol. 34: 351-372.

Marti T., Takio K., Walsh K.A., Terzi G. and Truman J.W. 1987. Microanalysis of the amino acid sequence of the eclosion hormone from the tobacco hornworm *Manduca sexta*. FEBS Lett. 219: 415-418.

Morton D.B. and Truman J.W. 1985. Steroid regulation of the peptide-mediated increase in cyclic GMP in the nervous system of the hawkmoth, *Manduca sexta*. J.Comp. Physiol. A. 157: 423-432.

Morton D.B. and Truman J.W. 1986. Substrate phosphoprotein availability regulates eclosion hormone sensitivity in an insect CNS. Nature 323: 264-267.
Morton D.B. and Truman J.W. 1988a. The EGPs - the eclosion hormone and cyclic GMP regulated phosphoproteins. I. Appearance and partial characterization in the CNS of *Manduca sexta*. J. Neurosci. 8: 1326-1337.
Morton D.B. and Truman J.W. 1988b. The EGPs - the eclosion hormone and cyclic GMP regulated phosphoproteins. II. Regulation of appearance by the steroid hormone 20-hydroxyecdysone in *Manduca sexta*. J. Neurosci. 8: 1338-1345.
Reynolds S.E. 1977. Control of cuticle extensibility in the wings of adult *Manduca* at the time of eclosion: Effects of eclosion hormone and bursicon. J. Exp. Biol. 70: 27-37.
Schwartz L.M. and Truman J.W. 1982. Peptide and steroid regulation of muscle degeneration in an insect. Science 215: 1420-1421.
Schwartz L.M. and Truman J.W. 1984. Cyclic GMP may serve as a second messenger in peptide-induced muscle degeneration in an insect. Proc. Natl. Acad. Sci. U.S.A. 81: 6718-6722.
Terzi G., Truman J.W. and Reynolds S.E. 1988. Purification and characterization of eclosion hormone from the moth, *Manduca sexta*. J. Insect Physiol. 18: 701-707.
Truman J.W. 1971. Physiology of insect ecdysis. I. The eclosion behavior of saturniid moths and its hormonal release. J. Exp. Biol. 54: 805-814.
Truman J.W. 1978. Hormonal release of stereotyped motor programmes from the isolated nervous system of the Cecropia silkmoth. J. Exp. Biol. 74: 151-174.
Truman J.W. and Copenhaver P.F. 1989. The larval eclosion hormone neurones in *Manduca sexta*: identification of the brain-proctodeal neurosecretory system. J. Exp. Biol. 147: 457-470.
Truman J.W. and Riddiford L.M. 1970. Neuroendocrine control of ecdysis in silkmoths. Science 167: 1624-1626.
Truman J.W., Taghert P.H., Copenhaver P.F., Tublitz N.J., and Schwartz L.M. 1981. Eclosion hormone may control all ecdyses in insects. Nature 291: 70-71.

BIOCHEMISTRY AND ENDOCRINE REGULATION OF SEX PHEROMONE PRODUCTION IN THE HOUSEFLY AND GERMAN COCKROACH

Gary J. Blomquist[1] and Coby Schal[2]

[1]Department of Biochemistry University of Nevada, Reno, Nevada 89557-0014
[2]Department of Entomology, Cook College, Rutgers University New Brunswick, New Jersey 08903

INTRODUCTION

There has been a dramatic increase of interest in the biosynthesis of insect sex pheromones over the last decade (Prestwich and Blomquist, 1987). It now appears that many of the rather novel compounds that are components of insect sex pheromones are produced by the addition of a few ancillary enzymes to those of "normal" metabolism. For example, the carbon skeletons of many lepidopteran sex pheromones are produced from common fatty acids by a novel delta-ll desaturase and highly specific chain-shortening reactions (Bjostad et al., 1987). In the Coleoptera, sex pheromones are often produced by the use of one or two highly specific enzymes that convert dietary material, usually isopreniod, to active pheromones (Vanderwel and Oehlschlager, 1987). By using one or a few ancillary enzymes to alter the products of the usual lipid metabolic pathways or to alter dietary constituents, the insect requires much less genetic material than would be necessary to code for a complete set of enzymes that would be expressed only in the pheromone-producing tissue. This phenomenon appears to exist in the housefly, *Musca domestica,* and the German cockroach, *Blattella germanica,* as hormone control of several enzymes could account for alteration of the products of cuticular lipid biosynthesis to produce the female-specific sex pheromone components.

Studies in the early 1960's implicated juvenile hormone (JH) in the regulation of volatile sex pheromones in several cockroaches and beetles (Barth, 1965). The use of bioassays to monitor pheromone production in these studies, however, made it difficult to quantify levels of pheromones and impossible to determine whether regulation occurred at the level of biosynthesis or release. Studies using radiochemical techniques to monitor sex pheromone production in the female housefly demonstrated that sex pheromone biosynthesis was induced by ecdysteroids produced by the maturing ovary. In cockroaches, beetles, flies, and some moths, the same hormones that control vitellogenesis also regulate sex pheromone production, thus coordinating egg maturation with mating. Recent work in several species of Lepidoptera has demonstrated that a neuropeptide, called pheromone biosynthesis activating neuropeptide (PBAN), induces sex pheromone production (Raina and Menn, 1987). There is considerable interest in the hormonal control of enzymes that produce sex pheromones, and a number of laboratories are currently addressing this problem. Results from work on the housefly and the German cockroach are discussed herein.

BIOSYNTHESIS AND ENDOCRINE REGULATION OF SEX PHEROMONE PRODUCTION IN THE HOUSEFLY

A sex pheromone was first demonstrated in the housefly by Rogoff et al. (1964),

Molecular Insect Science, Edited by
H. H. Hagedorn *et al.,* Plenum Press, New York, 1990

identified as Z9-23:Hy [(Z)-9 tricosene, muscalure] (Carlson et al., 1971), and shown to attract males in an olfactometer at doses as low as 0.07 µg (Adams et al., 1984). The C_{23} epoxide (9,10-epoxytricosane), C_{23} ketone [(Z)-14-tricosen-10-one] (Uebel et al., 1978) and methylalkanes (Uebel et al., 1976; Rogoff et al., 1980) are also part of the pheromone. Adams and Holt (1987) showed that each of these pheromone components had different roles in male courtship behavior. Z9-23:Hy increased male mating strike activity toward females and other males. The epoxide and ketone decreased the number of homosexual mating strikes, thus acting as a sex recognition factor. The methylalkanes acted as arrestants and increased the amount of time that a male spent with a treated model.

Females with previtellogenic ovaries at stage 2 or 3 (0-2 days post emergence) do not have detectable amounts of any of the C_{23} sex pheromone components (Dillwith et al., 1983). The C_{23} sex pheromone is first detected at stage 4 and steadily increases to a maximum by stage 8.

The sex pheromone of the housefly consists of modified cuticular lipids. Newly emerged males and females have similar cuticular lipid profiles: the major hydrocarbon components are (Z)-9-alkenes of 27 carbons and longer. From stages 4 through 8 (vitellogenic stages), both the percentage of alkenes and the percentage of Z9-27:Hy in the alkene fraction decrease, while the percentage of Z9-23:Hy increases. As Z9-23:Hy is readily converted to both the C_{23} epoxide and ketone (Blomquist et al., 1984b; Ahmad et al., 1987), the increase in Z9-23:Hy is mirrored by a concomitant increase in the C_{23} epoxide and ketone. The incorporation of radioactivity from labeled acetate into the C_{23} sex pheromone components follows a pattern consistent with that for the amount of pheromone present and emphasizes the role that the maturing ovary plays in initiating sex pheromone production (Dillwith et al., 1983).

Because pheromone production is correlated with ovarian development, it is possible that both processes are regulated by a common factor, as has been demonstrated in some cockroaches and beetles, where JH induces vitellogenesis and sex pheromone production. Alternatively, a product of the developing ovary could initiate pheromone production. To determine which process occurs in the housefly, experiments were performed to examine the effect of removing the corpus allatum-corpus cardiacum (CA-CC) complex and of ovariectomy on sex pheromone production. While removal of the ring gland had no effect on preventing sex pheromone production, flies ovariectomized within 6 hours of emergence did not produce detectable amounts of any of the C_{23} pheromone components. If a product from the developing ovary were involved in inducing sex pheromone synthesis or the ovary mediated in regulation by other systems, then reimplanting ovaries into ovariectomized insects should restore pheromone production. When insects ovariectomized within 6 hours of emergence received ovaries on day 4 and were then maintained for 3 days, they produced C_{23} sex pheromone components at levels similar to a female with stage 5 ovaries. All of the implanted ovaries became vitellogenic with stages varying from 4-6 (Adams et al., 1984a, b). Thus, it was concluded that either the ovary mediated in pheromone production or a product from the ovary stimulated sex pheromone biosynthesis.

Because females of some insects have repeated reproductive cycles and mate only during specific periods within each cycle, it has been suggested that pheromone production might be humorally mediated. Engelmann (1960) first suggested that products from the corpora allata mediated receptivity in the female cockroach *Leucophaea maderae*. Subsequent studies with the Cuban cockroach, *Byrostria fumigata* (Barth, 1961, 1962), showed that females required functional CA to produce sex pheromone. Furthermore, JH was shown to mediate sex pheromone production in other insects, including the cockroaches *Pycnoscelus indicus* (Barth, 1965), and *Supella longipalpa* (Smith and Schal, 1990a), the moth *Pseudaletia unipuncta* (Cusson and McNeil, 1989) and various beetles (Vanderwel and Oehlschlager, 1987) including *Tenebrio molitor* (Menon, 1970, 1976) and *Ips paraconfusus* (Hughes and Renwick, 1977; Borden et al., 1969). As JH induces vitellogenesis and regulates ovarian development and other reproductive events in many insect species, the observation that JH also regulates sex pheromone production suggested that the same hormone may be used to coordinate different reproductive events (Blomquist and Dillwith, 1983).

In many Diptera, ecdysteroids have been shown to play a role in regulating reproductive processes, including vitellogenin synthesis (Adams et al., 1985; Hagedorn, 1985; Bownes, 1986). To determine if ecdysteroids can restore sex pheromone production in ovariectomized flies, a single dose of 20-hydroxyecdysone (20-HE) was injected into insects that had been ovariectomized within 6 hr of emergence. At 16 and 24 hrs after 20-

HE treatment, flies produced all of the C_{23} pheromone components. Doses as low as 50 ng injected repeatedly (20-HE has a half life of 16 minutes in the housefly [Adams et al., 1985]) at regular intervals into ovariectomized insects induced sex pheromone production.

Vitellogenin is normally produced only by females, but the injection of 20-HE into male *Drosophila melanogaster* (Bownes, 1982) and *Sarcophaga bullata* (Huybrechts and De Loof, 1977, 1981) induced vitellogenin production. Thus, experiments were performed to determine whether male houseflies, which normally do not produce any of the C_{23} sex pheromone components, could be induced to produce female-specific pheromones after ovary implants or injections of 20-HE. Implanting ovaries or injecting 20-HE into male houseflies induced sex pheromone production, including all of the C_{23} pheromone components, in a dose and time dependent manner (Blomquist et al., 1984a). Control males did not produce any of the C_{23} pheromone components. Thus, these data demonstrate that males can biosynthesize female sex pheromone, and that treatment with 20-HE alters the production of cuticular components such that Z9-23:Hy becomes a major product. Males and females of all ages readily metabolize Z9-23:Hy applied to their surfaces to the corresponding epoxide and ketone (Blomquist et al., 1984b) via a cytochrome P450 polysubstrate mono-oxygenase (Ahmad et al., 1987).

Further evidence that ecdysteroids directly induce sex pheromone production comes from studies in which isolated male housefly abdomens were injected with 20-HE, and assayed 24 hours later for their ability to produce Z9-23:Hy. The results showed that radioactivity was incorporated into Z9-23:Hy (Blomquist and Adams, unpublished data).

Female houseflies that had the CA-CC complex removed within 6 hours of eclosion still produced Z9-23:Hy, C23 epoxide and C23 ketone when assayed at 4 to 6 days postemergence (Blomquist and Adams, unpublished observations) and also developed ovaries to stage 4. This suggests that JH is not directly required for pheromone production. More recent studies were performed to determine if JH, while unable to induce sex pheromone biosynthesis by itself, might synergize the effect of ecdysteroids in inducing sex pheromone production. The results of this study (Blomquist and Adams, unpublished), in which varying doses of a JH analogue and 20-HE were administered at 18 hour intervals further indicate that JH does not play a role in inducing sex pheromone biosynthesis.

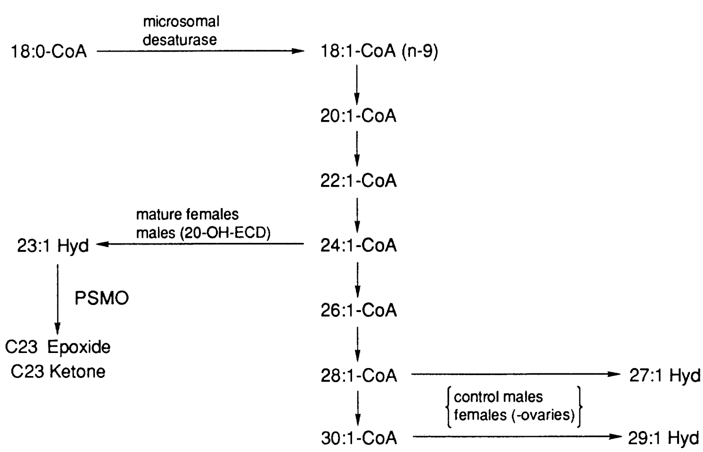


Fig. 1. Biosynthesis of alkenes by houseflies. Mature females and males treated with 20-hydroxyecdysone (20-OH-ECD) produce Z9-23:Hy whereas newly emerged females, control males and ovariectomized females produce alkenes of 27 carbons and longer.

Sex pheromone-producing tissues in insects vary in complexity from individual unicellular glands distributed throughout the integument to elaborate internal cellular glands (Percy-Cunningham and MacDonald, 1987). Schlein et al. (1980) reported that unicellular glands on the legs of female houseflies synthesized the sex pheromone. Subsequent work using isolated tissue and radio-tracer techniques demonstrated that the sex pheromone com-

ponents of the housefly were synthesized by epidermal tissue (Dillwith et al., 1981; Dillwith and Blomquist, 1982) as expected for modified components of cuticular lipid (Blomquist et al., 1987.

Biosynthesis of Z9-23:Hy in female houseflies occurs by the microsomal elongation of oleoyl-CoA to a 24 carbon fatty acyl moiety which is then converted to an alkene one carbon shorter (Fig. 1) (Blomquist et al., 1987). The elongation of oleoyl-CoA to 20-, 22- and 24-carbon monounsaturated acyl derivatives requires malonyl-CoA and either NADPH or NADH (de Renobales et al., 1986; Vaz et al., 1988). Microsomes prepared from abdomens have higher elongation activity than those prepared from either thorax or head tissue (Vaz et al., 1988), and microsomes prepared from abdominal epidermal tissue have higher activity than those prepared from fat body tissue. Preliminary data using microsomal preparations from 2 day old males and 1-day-old females (both of which produce alkenes of 27 carbons and longer) versus 3 or 4 day old females or males treated with 20-HE (in which the major hydrocarbon produced is Z9-23:Hy) indicate differences in the rates of elongation of 18:1-CoA, 20:1-CoA, 22:1-CoA and 24:1-CoA. In both situations, 18:1-CoA is readily elongated, but when the insect is producing primarily Z9-27:Hy (control males or previtellogenic females), larger amounts of 26:1 fatty acid and 28:1 fatty acid are produced than when the insect is producing Z9-23:Hy. This indicates that the elongation reactions play a role in determining the chain length of the hydrocarbon product. Furthermore, when 24:1-CoA is used as the substrate, it is much more efficiently elongated by microsomes prepared from insects which are synthesizing alkenes of 27 carbons and longer (males and previtellogenic females) than those which produce Z9-23:Hy (Vaz et al., 1989). A possible explanation for the differences in chain length specificity of the housefly elongation system under conditions where ecdysteroids are present (mature females) or absent (males, previtellogenic females) is that there are two or more chain-elongation systems, each with a different chain length specificity. There could be one enzyme or set of enzymes that elongates fatty acyl CoAs of 18:1 to 24:1, and then a different elongation enzyme or set of enzymes that elongate 24:1 to 28:1 and 30:1. This possibility is currently being examined.

BIOSYNTHESIS AND ENDOCRINE REGULATION OF SEX PHEROMONE PRODUCTION IN THE GERMAN COCKROACH

Roth and Willis (1952) demonstrated that *B. germanica* females possess a substance in the cuticular wax that could elicit courtship wing-raising displays in males. Unlike the American cockroach, *Periplaneta americana*, in which the pheromone could be absorbed to filter paper or feces and would elicit wing- raising in an olfactometer, the pheromone of *B. germanica* stimulated male courtship only upon contact (Roth and Willis, 1952). Four components have been identified, all of which possess a 3,11-dimethyl ketone skeleton: 3,11-dimethyl-2-nonacosanone (Nishida et al., 1974), 3,11-dimethyl-2-heptacosanone (Schal et al., 1990b), 29-hydroxy-3-11-dimethyl-2-nonacosanone (Nishida et al., 1976), and 29-oxo-3,11-dimethyl-2-nonacosanone (Nishida and Fukami, 1983). The methyl ketone fraction of male extracts did not contain either the C_{27} or C_{29} component and did not elicit sexual responses in males (Nishida and Fukami, 1983; Schal et al., unpublished).

The ratio of 3,11-dimethyl-2-nonacosanone to 3,11-dimethyl-2-hepta-cosanone in surface washes of sexually receptive females was approximately 60:40, while the other two components comprised only minor fractions of the mass (Schal et al., 1990b). The alcohol was more active, however, and the 29-oxo methyl ketone was less active than 3,11-dimethyl-2-nonacosanone (Nishida and Fukami, 1983), and the C27 methyl ketone was at least an order of magnitude less effective at eliciting male wing-raising behavior (Schal et al., 1990b). Fifty and 75 percent of males responded to 1 and 3.5 ng of 3,11- dimethyl-2-nonacosanone, respectively. There are also various unknowns in the TLC methyl ketone fraction which may comprise other pheromone components. Moreover, presence of 3,11-dimethylhentriacontane in cuticular extracts of females (Carlson and Brenner, 1988), together with the suggestion that the methyl ketones are formed through oxidation of the respective alkanes by a 3,11-dimethyl specific enzyme (Jurenka et al., 1989), would predict that 3,11-dimethyl-2-hentriacontanone will be found as another pheromone component.

Activity of the sex pheromone blend on behavior of *B. germanica* was strikingly different from blends in other insects (e.g., Lepidoptera) including the housefly. Each component alone could elicit the complete courtship wing-raising response in male *B. ger-*

manica (Nishida and Fukami, 1983; Schal et al., 1990b), whereas in most insects deletion of components reduces sexual responses (review: Tamaki, 1985). Combining the two methyl ketones did not enhance their activity, but rather their combined effectiveness was intermediate between the activity of each component alone (Schal et al., 1990b). Likewise, cuticular hydrocarbons, which are qualitatively identical in nymphs and adult males and females (Jurenka et al., 1989), did not enhance the activity of the pheromone.

Behavioral assays of males, using excised female antennae from various age insects, showed that the antennae from newly emerged females and vitellogenic females elicited wing-raising in most males, but male response was low to antennae of previtellogenic females (Nishida and Fukami, 1983; Schal et al., 1990b). Gas-chromatographic analyses of female cuticular washes showed, however, that the amounts of both C_{27} and C_{29} methyl ketones, and the C_{27} and C_{29} methyl alcohol gradually increased after emergence, in relation to oocyte maturation, until ovulation and oviposition (day 9 grouped females or day 12 isolated females) (Schal, 1988; Schal et al., 1990a, 1990b). This discrepancy between behav-ioral and analytical results prompted Schal et al. (1990b) to hypothesize that newly emerged females might contain other factors that can induce wing-raising in males. Following ovipo-sition in mated females, the mass of 3,11-dimethyl-2-nonacosanone remained relatively unchanged through approximately 20 days of "pregnancy" (the female retains the egg case externally, attached to the vestibulum), during which time oocyte development is arrested. Pheromone production and oocyte maturation resume after the egg case hatches (Schal et al., 1990a; unpublished). In virgin females, the first infertile egg case is aborted prematurely (Gadot et al., 1989b), and both oocyte maturation and pheromone production resume earlier than in mated females (Schal et al., 1990a). Thus, a correlation between pheromone production and oocyte maturation was established.

The *in vivo* incorporation of radioactivity from labeled propionate into the methyl ketone fraction followed a pattern, as in the housefly, that was consistent with that for mass (Schal, Burns, Gadot, Jurenka, Blomquist, unpublished). The greatest incorporation of radioactivity corresponded to the most rapid oocyte development, while during pregnancy little ^{14}C was incorporated into pheromone components. The patterns of pheromone biosyn-thesis over time was identical to that of JH biosynthesis by the CA *in vitro* (Gadot et al., 1989a). As a function of basal oocyte length, the pattern of pheromone synthesis (Schal, Burns, Gadot, Jurenka, Blomquist, unpublished) corresponded well with that of JH biosynthesis in both virgin and mated females (Gadot et al., 1989a). Thus it appeared that JH, which plays a key role in reproduction in B. germanica (see Kunkel, 1981; Gadot et al., 1989a), also controls pheromone production.

In the cockroach *Byrsotria fumigata*, allatectomy inhibited both pheromone production and mating (Barth, 1962), but mating occurred in allatectomized females treated with pheromone (Roth and Barth, 1964). In *Supella longipalpa* both volatile sex pheromone production and calling behavior were suppressed by allatectomies (Smith and Schal, 1990a). In both species, treatment with JH or a JH analog induced pheromone production (Bell and Barth, 1970; Smith and Schal, 1990a). In *B. germanica*, allatectomy, decapitation, head-ligation, or inhibition of the CA with precocene or with egg case implants into the vestibulum, all reduced, but did not eliminate, pheromone production (Schal et al., 1989a). Reimplantations of CA and treatment of CA-deprived- or CA- inhibited-females with a JH analog induced pheromone production in a dose-dependent relationship (Schal et al., 1990a). Thus, it appears that pheromone production in *B. germanica* is related to JH, and it proceeds, albeit at low rates, in CA-deprived females. Long-term allatectomy studies revealed that the rates of pheromone synthesis (radio-tracer studies) and accumulation (gas chromatographic studies) were low in allatectomized females. By day 30, however, they contained equal amounts of pheromone to sham-operated females (Schal, Burns, Blomquist, unpublished). Differences between head-ligated and allatectomized females (Schal et al., 1990a) suggest that this non-JH-regulated process might be related to feeding.

Feeding appeared to affect pheromone production in two ways. First, by affecting CA activity, feeding or starvation could influence pheromone production. Weaver (1984) showed that food and water were essential for stimulation of CA activity and oocyte growth in *P. americana*. Protein-deprived *Diploptera punctata* females had lower rates of JH synthesis in vitro and they oviposited later than control females (Woodhead and Stay, 1989). In *B. germanica,* starvation completely suppressed JH synthesis (Schal, unpublished). Females starved from day 0, with access to water only, accumulated less 3,11-dimethyl-2-nonacosanone by day 15 than allatectomized females (Schal et al., 1990a). Starved females,

induced by treatment with a juvenile hormone analog, produced significantly less pheromone than either induced or control fed females, indicating that, in addition to its effect on JH, feeding might directly influence pheromone production. Because allatectomized females accumulated greater amounts of 3, 11-dimethylnonacosane (presumed pheromone precursor) than sham-operated females, and starved females do not, our working hypothesis is that starvation removes or redirects essential substrates for pheromone synthesis.

Thus, two mechanisms appear to regulate sex pheromone production in *B. germanica*: a JH-dependent mechanism which may induce conversion of a late precursor (possibly hydrocarbon) to pheromone (see below), and a feeding-related mechanism which provides substrates for the synthesis of hydrocarbons.

Regulation of the contact sex pheromone of *B. germanica* appears to differ significantly from regulation of volatile sex pheromones in other cockroach species. Using behavioral assays, allatectomy was shown to completely suppress pheromone production in *B. fumigata* (Barth, 1962), *P. americana* and *Pycnoscelus indicus* (cited in Barth and Lester, 1973). In *Supella longipalpa,* males normally respond in an olfactometer to 0.001 of a female equivalent (Liang and Schal, 1990), but males did not respond to extracts of whole allatectomized females and female calling behavior was also suppressed (Smith and Schal, 1990a).

B. germanica also respond differently to induction by JH or JH analog than do species with volatile pheromones. Both low and high doses of JHA induced pheromone production in allatectomized and intact *B. germanica* females (Schal et al., 1990a). Allatectomized *B. fumigata, P. americana,* and *S. longipalpa* females treated with low dosages of JH analog produced pheromone (Bell and Barth, 1970; Barth and Lester, 1973; Smith and Schal, 1990a), but exposure of both *B. fumigata* (Bell and Barth, 1970) and *S. longipalpa* (Smith and Schal, 1990a) to high doses of JH analog delayed or suppressed pheromone production and calling behavior (in *S. longipalpa*). The hypothesis that high titers of JH turn off pheromone production because they are normally associated with mating (Bell and Barth, 1970) does not explain this phenomenon, as CA from virgin and mated *S. longipalpa* females attained the same levels of JH synthesis *in vitro* (Smith et al., 1989), and pheromone production is not suppressed in virgins when the CA are maximally active (Smith and Schal, 1990a).

Also, in *B. germanica*, unlike *B. fumigata* and *S. longipalpa*, mating does not inhibit pheromone production; in fact, CA activity increased after mating (Gadot et al., 1989a) and both mass of the pheromone (Schal, 1988; unpublished) and its synthesis (Schal, Burns, Gadot, Jurenka, Blomquist, unpublished) increase after mating. Pheromone is also produced in mated *B. germanica* females in the second ovarian cycle (Schal et al., 1990a), whereas in *S. longipalpa,* mating suppresses pheromone production and calling for at least 12 ovarian cycles (Smith and Schal, 1990b). Thus, as with JHA applications, a direct positive relationship appears to exist between endogenous JH and pheromone production in *B. germanica:* as JH and JHA increase, so does pheromone production.

Regulation of contact pheromones in *B. germanica* thus appears to be more similar to regulation of pheromone production in the housefly (above) than to other cockroaches (see Schal and Smith, 1990). As in *B. germanica*, both mass and synthetic rate of the sex pheromone increased in vitellogenic and post-vitellogenic female houseflies (Dillwith et al., 1983) and accumulation of pheromone on the cuticle continued after mating. As in *B. germanica,* pheromone production in the housefly was induced by both low and high doses of the regulatory hormone (JH and 20-HE, respectively).

It is possible, as in the housefly, that ovarian factors mediate the regulation of pheromone production. Ovariectomy did not eliminate pheromone production in the ovoviviparous *B. fumigata* (Barth, 1962) and in the oviparous *S. longipalpa* (Smith and Schal, 1990a). However, because neither study measured both pheromone production and JH synthesis in ovariectomized females, it is not known whether the CA were activated or whether pheromone production occurred in the absence of JH. For example, in the cockroaches *Nauphoeta cinerea* and *D. punctata* removal of the ovaries abolished both the activation and the cyclical activity of the CA (Lanzrein et al., 1981; Stay et al., 1983). In ovariectomized *B. germanica* the C_{29} methyl ketone pheromone accumulated on the cuticle (Schal, 1988) and the CA were activated and exhibited at least one cycle of activity, as established by the *in vitro* radiochemical assay (Gadot et al., 1990). Thus, in all cock-

roaches examined to date, ovariectomy did not suppress pheromone production, indicating either that the CA can be activated to normal levels or that very low titers of JH can induce pheromone production. The latter was shown to be true for *S. longipalpa* in which CA activity in vitro was approximately 5% of peak activity when volatile pheromone production was first detected in behavioral assays (Smith et al., 1989; Smith and Schal, 1990a).

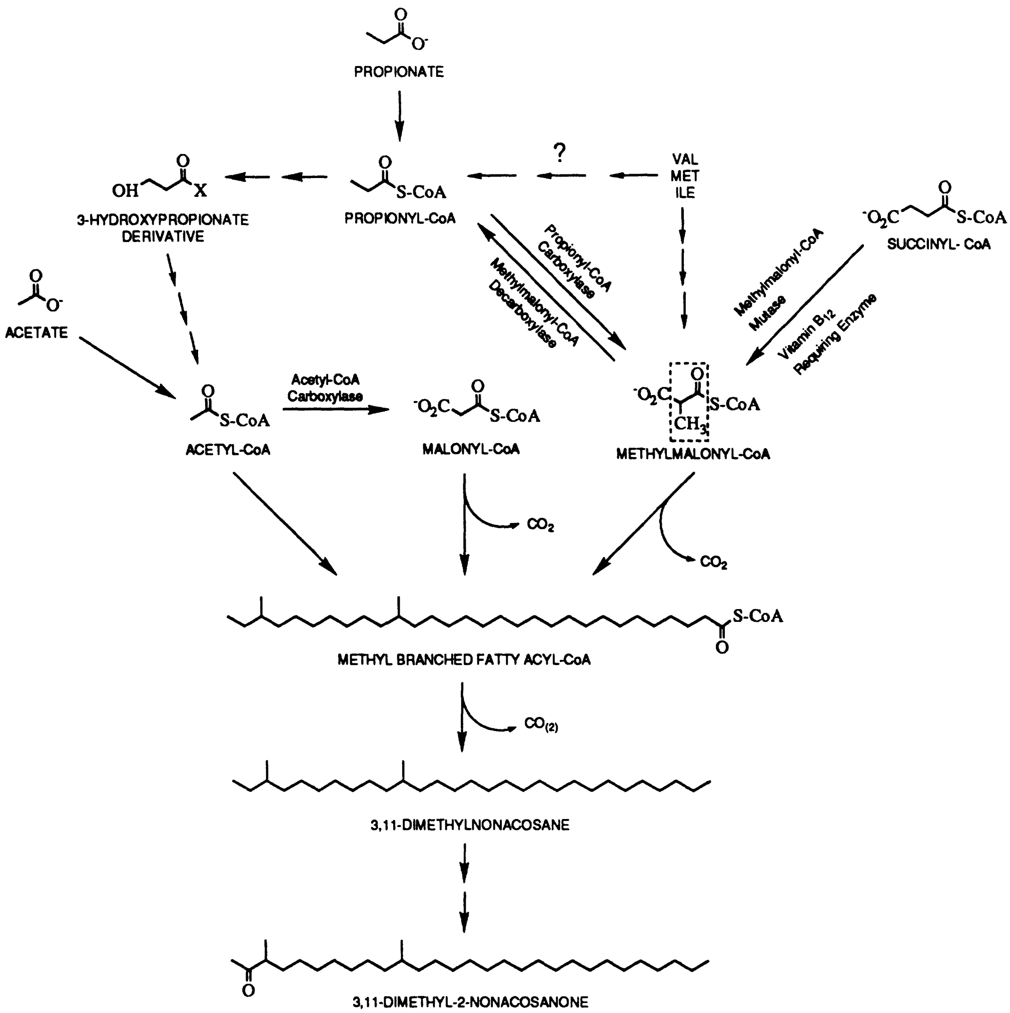


Fig. 2. Proposed biosynthetic pathway for the major female sex pheromone component of the German cockroach, *Blattella germanica*.

Whole females and isolated body parts of sexually mature females elicited the complete courtship wing-raising response in males (Roth and Willis, 1952; Nishida and Fukami, 1983). As in the housefly, determination of the site of pheromone synthesis by behavioral assays or by GC may be misleading. Preliminary data showed that 33% of extractable cuticular pheromone occurred on the wings of *B. germanica* females (24% on the abdomen), but it is likely that radiotracer techniques will show that pheromone is synthesized elsewhere (most probably by abdominal epidermal cells) and concentrated on the wings.

The major hydrocarbon component of *B. germanica* females is an isomeric mixture of 3,7-, 3,9- and 3,11-dimethylnonacosane (Augustynowicz et al., 1987; Carlson and Brenner, 1988; Jurenka et al., 1989). Jurenka et al. (1989) and Chase et al. (1990) suggest that the production of the 3,11-dimethyl ketone pheromone could result from the oxidation of its hydrocarbon analog by a sex-specific polysubstrate mono-oxygenase (Fig. 2). Preliminary studies indicate that radiolabel from [10,11-^{3}H]dimethylnonacosane applied to females at times when they are actively producing sex pheromone is recovered in the methyl ketone pheromone fraction (Chase, Schal, Touhara, Prestwich and Blomquist, unpublished). Also, the cuticular mass of both the dimethylnonacosane and dimethylheptacosane, the presumed precursors of 3,11-dimethyl-2-nonacosanone and 3,11-dimethyl-2-heptacosanone, respectively, remained relatively unchanged during the ovarian cycle, except for slight increases before the onset of pheromone synthesis, and sharp declines during the most active phase of pheromone synthesis. Other hydrocarbons (e.g., n-nonacosane) increased gradually over time with no apparent relation to the gonotrophic cycle (Schal, unpublished). Finally, in allatectomized females the mass of dimethylalkanes increased over time (Schal, Burns, and Blomquist, unpublished) and presumably contributed to greater synthesis and accumulation of pheromone (see above). These data lend support to the above hypothesis.

The precursors and directionality of biosynthesis of the methyl branched cuticular hydrocarbons were examined by Chase et al. (1990). The amino acids [G-^{3}H]valine, [4,5-^{3}H]isoleucine, and [3,4-$^{14}C_2$]methionine labeled the hydrocarbon fraction in a manner indicating that the carbon skeletons of all three amino acids serve as precursors to methylmalonyl-CoA, the methyl branch group donor (Fig. 2). The incorporation of [1,4-$^{14}C_2$]- and [2,3-$^{14}C_2$]succinates into the hydrocarbon and acylglycerol plus polar lipid fractions indicates that succinate also serves as a precursor to methylmalonyl-CoA. Carbon-13 NMR analyses showed that [1-^{13}C]propionate labeled the carbon adjacent to the tertiary carbon, and, for the 3,x-dimethylalkanes, that carbon-4 but not carbon-2 was enriched. This indicates that the methyl branching groups of the 3, x-dimethylalkanes are inserted early in the chain elongation process. [3,4,5-$^{13}C_3$]Valine labeled the methyl and tertiary carbons as well as the carbons adjacent to the tertiary carbon of the methyl branched alkanes. These data, taken together, indicate that the methyl branch is formed early in chain elongation by the insertion of methylmalonyl-CoA units derived from either branched chain amino acids or succinate. Subsequent acetate units are added, the methyl branched fatty acyl group is converted to hydrocarbon, and finally the 3,11-dimethylalkane oxidized to the corresponding methyl ketone (Fig. 2) (Chase et al., 1990).

SUMMARY

The sex pheromones of the female housefly and German cockroach consist of modified cuticular lipid components and are regulated by the same hormones that are involved in other reproductive events. In the female housefly, the sex pheromone components Z9-23:Hy, C_{23} epoxide, and C_{23} ketone first appear when the female becomes vitellogenic and are induced by ecdysteroids which are produced in the maturing ovary. Ovariectomy prevents sex pheromone production whereas reimplantation of ovaries or treatment with 20-HE restores sex pheromone production. Apparently 20-HE induces changes in the chain length specificities of the enzymes that are involved in elongating fatty acyl-CoAs and the step that reductively converts long chain fatty acyl-CoAs to hydrocarbon. The Z9-23:Hy formed under the influence of ecdysteroids is then converted to the corresponding epoxide and ketone via polysubstrate monooxygenase (PSMO) activity, which does not appear to be under endocrine regulation. In the German cockroach, males and females of all ages produce 3,11-dimethylnonacosane as a major cuticular lipid component, which is then apparently converted to the methyl ketone pheromone components in females under the influence of juvenile hormone (JH). Ovariectomy of German cockroaches does not inhibit sex pheromone production, but allatectomy partially depresses the synthesis and accumulation of sex pheromone. Juvenile hormone analogs induce pheromone production in both allatectomized and intact females.

ACKNOWLEDGMENTS

Supported in part by NSF grant DCB-890088 to Gary J. Blomquist, and NIH grant HD-21891 to Coby Schal.

REFERENCES

Adams, T. S. and Holt, G. G. 1987. Effect of pheromone components when applied to different models on male sexual behavior in the housefly, *Musca domestica*. J. Insect Physiol. 33: 9-18.

Adams, T. S., Dillwith, J. W. and Blomquist, G. J. 1984a. The role of 20-hydroxyecdysone in housefly sex pheromone biosynthesis. J. Insect Physiol. 30: 287-294.

Adams, T. S., Holt, G. G. and Blomquist, G. J. 1984b. Endocrine control of pheromone biosynthesis and mating behavior in the housefly, *Musca domestica*. pp 441-456 in: "Advances in Invertebrate Reproduction 3." Engels, W. ed. Elsevier, Amsterdam.

Adams, T. S., Hagedorn, H. H. and Wheelock, G. D. 1985. Hemolymph ecdysteroid in the housefly, *Musca domestica*, during oogenesis and its relationship with vitellogenin levels. J. Insect Physiol. 31: 91-97.

Ahmad, S., Kirkland, K. E. and Blomquist, G. J. 1987. Evidence for a sex pheromone metabolizing cytochrome P-450 monooxygenase in the housefly. Arch. Insect Biochem. Physiol. 6: 121-140.

Augustynowicz, M., Malinski, E., Warnke, Z., Szafranek, J., and Nawrot, J. 1987. Cuticular hydrocarbons of the German cockroach, *Blattella germanica*. Comp. Biochem. Physiol. 86B: 519-523.

Barth, R. H. 1961. Hormonal control of sex attractant production in the Cuban cockroach. Science 133: 1598-1599.

Barth, R.H., Jr. 1962. The endocrine control of mating behavior in the cockroach *Byrsotria fumigata*. Gen. Comp. Endocr. 2: 53-69.

Barth, R. H. 1965. Insect mating behavior: Endocrine control of a chemical communication system. Science 149: 882-883.

Barth, R.H., Jr., and Lester, L.J. 1973. Neuro-hormonal control of sexual behavior in insects. Ann. Rev. Entomol. 18: 445-472.

Bell, W.J., and Barth, R.J., Jr. 1970. Quantitative effects of juvenile hormone on reproduction in the cockroach *Byrsotria fumigata*. J. Insect Physiol. 16: 2303-2313.

Bjostad, L. B., Wolf, W. A. and Roelofs, W. L. 1987. Pheromone biosynthesis in lepidopterans: Desaturation and chain shortening. pp 77-120 in: "Pheromone Biochemistry." Prestwich, G. D. and Blomquist, G. J., eds. Academic Press, New York, N. Y.

Blomquist, G. J., Adams, T. S. and Dillwith, J. W. 1984a. Induction of female sex phermone production in male houseflies by ovarian implants or 20-hydroxyecdysone. J. Insect Physiol. 30: 295-302.

Blomquist, G. J., Dillwith, J. W. and Pomonis, J. G. 1984b. Sex pheromone of the housefly: Metabolism of (Z)-9-tricosene to (Z)-9,10-epoxytricosane and (Z)-14-tricosene-10-one. Insect Biochem. 14: 279-284.

Blomquist, G. J. and Dillwith, J. W. 1983. Pheromones: Biochemistry and Physiology. pp 527-542 in: "Endocrinology of Insects." Downer, R. G. H. and Laufer, H., eds. Vol 1. Alan R. Liss, New York, N. Y.

Blomquist, G. J., Dillwith, J. W. and Adams, T. S. 1987. Biosynthesis and endocrine regulation of sex pheromone production in Diptera. pp 217-250 in: "Pheromone Biochemistry." Prestwich, G. D. and Blomquist, G. J., eds. Academic Press, Inc. New York, N.Y.

Borden, J. H., Nair, K. K. and Slater, C. E. 1969. Synthetic juvenile hormone: Induction of sex pheromone production in *Ips confusus*. Science 166: 1626-1627.

Bownes, M. 1982. The role of yolk polypeptide synthesis by male and female fat bodies of *Drosophila melanogaster*. J. Insect Physiol. 28: 317-328.

Bownes, M. 1986. Expression of the genes coding for vitellogenin (yolk protein). Annu. Rev. Entomol. 31: 507-531.

Carlson, D.A., and Brenner, R.J. 1988. Hydrocarbon-based discrimination of the North American *Blatella* cockroach species using gas chromatography. Ann. Entomol. Soc. Am. 81: 711-723.

Carlson, D. A., Mayer, M. S., Silhacek, D. L., Beroza, M. and Bierl, B. A. 1971. Sex attractant pheromone of the housefly: Isolation, identification and synthesis. Science 174: 76-78.

Chase, J., Jurenka, R.A., Schal, C., Halarnkar, P.P., and Blomquist, G.J. 1990. Biosynthesis of methyl branched hydrocarbons: Precursors of the female contact sex pheromone of the German cockroach *Blattella germanica*. Insect Biochem. 20: 149-156..

Cusson, J., and McNeil, J.N. 1989. Involvement of juvenile hormone in the regulation of pheromone release activities in a moth. Science 243: 210-212.
de Renobales, M., Wakayama, E, J., Halarnkar, R. C., Reitz, R. C. and Blomquist, G. J. 1986. Inhibition of hydrocarbon biosynthesis in the housefly be 2-octadecynoate. Arch. Insect Biochem. Physiol. 3: 75-86.
Dillwith, J. W., Adams, T. S., and Blomquist, G. L. 1983. Correlation of housefly sex pheromone production with ovarian development. J. Insect Physiol. 29: 377-386.
Dillwith, J. W. and Blomquist, G. J. 1982. Site of sex pheromone biosynthesis in the female housefly, *Musca domestica*. Experientia 38: 471-473.
Dillwith, J. W., Blomquist, G. J. and Nelson, D. R. 1981. Biosyntheisis of the hydrocarbon components of the sex pheromone of the housefly, *Musca domestica*. Insect Biochem. 11: 247-253.
Engelmann, F. 1960. Mechanisms controlling reproduction in two viviparous cockroaches (Blataria). Ann. N. Y. Acad. Sci. 89: 516-536.
Gadot, M., Chiang, A-S., and Schal, C. 1989a. Farensoic acid-stimulated rates of juvenile hormone biosynthesis during the gonotrophic cycle of *Blattella germanica*. J. Insect Physiol. 35: 537-542.
Gadot, M., Burns, E., and Schal, C. 1989b. Juvenile hormone biosynthesis and oocyte development in adult female *Blattella germanica*: Effects of grouping and mating. Arch. Insect Biochem. Physiol. 11: 189-200.
Gadot, M., Chiang, A-S., Burns, E. and Schal, C. 1990. Patterns of corpora allata activity in ovariectomized Blattella germanica. pp 321-324 in: "Insect Neurochemistry and Neurophysiology." Borkovec, A.B. and Masler, E. P., eds. Humana Press, Clifton New Jersy.
Hagedorn, H. H. 1985. The role of ecdysteroids in reproduction. pp 205-262 in: "Comprehensive Insect Physiology, Biochemistry, and Pharmacology." Vol 8. Kerkut, G. A. and Gilbert, L. I., eds. Pergamon Press, Oxford.
Hughes, P. R. and Renwick, J. A. A. 1977. Neural and hormonal control of pheromone biosynthesis in the bark beetle *Ips paraconfusus*. Physiol. Entomol. 2: 117-123.
Huybrechts, R. and De Loof, A. 1977. Induction of vitellogenin synthesis in male *Sarcophaga bullata* by ecdysterone. J. Insect Physiol. 23: 1359-1362.
Huybrechts, R. and De Loof, A. 1981. Effect of ecdysterone on vitellogenin concentration in haemolymph of male and female *Sarcophaga Bullata*. Int. J. Invert. Reprod. 3: 157-168.
Jurenka, R. A., Schal, C., Burns, E., Chase, J., and Blomquist, G. J. 1989. Structural correlation between the cuticular hydrocarbons and the female contact sex pheromone of the German cockroach *Blattella germanica*. J. Chem. Ecol. 15: 939-949.
Kunkel, J. G. 1981. A minimal model of metamorphosis-fat body competence to respond to juvenile hormone. pp 107-129 in: "Current Topics in Insect Endocrinology and Nutrition." Bhaskaran,G., Friedman, M. and Rodriguez, J., eds. Plenum Press, New York.
Lanzrein, B., Wilhelm, R., and Gentinetta, V. 1981. On relations between corpus allatum activity and oocyte maturation in the cockroach *Nauphoeta cinerea*. pp 149-160 in: "Regulation of Insect Development and Behaviour." Sehnal, F, Zabza, B., Menn, J J., and. Cymborowski, B, eds. Wroclaw Technical Univ. Press. Wroclaw, Poland.
Liang, D., and Schal, C. 1990. Effects of pheromone concentration and photoperiod on the behavioral response sequence to sex pheromone in the male brown-banded cockroach, *Supella longipalpa*. J. Insect Behav. 3: 218-223.
Menon, M. 1970. Hormone-pheromone relationships in the beetle, *Tenebrio molitor*. J. Insect Physiol. 16: 1123-1139.
Menon, M. 1976. Hormone-pheromone relationships of male *Tenebrio molitor*. J. Insect Physiol. 22: 1021-1023.
Nishida, R., and Fukami, J. 1983. Female sex pheromone of the German cockroach, *Blattella germanica*. Memoirs Coll. Agric. Kyoto Univ. 122: 1-24.
Nishida, R. Fukami, H., and Ishii, S. 1974. Sex pheromone of the German cockroach (*Blattella germanica*) responsible for male wing- raising: 3,11-dimethyl-2-nonacosanone. Experientia 30: 978-979.
Nishida, R., Sato, T., Kuwahara, Y., Fukami, H., and Ishii, S. 1976. Female sex pheromone of the German cockroach, *Blattella germanica*, responsible for male wing-raising. II. 29-hydroxy-3,11- dimethyl-2-nonacosanone. J. Chem. Ecol. 2:449-455.

Percy-Cunningham, J. and MacDonald, J. A. 1987. Biology and ultrastructure of sex pheromone-producing glands. pp 27-75 in: "Pheromone Biochemistry." Prestwich, G. D. and Blomquist, G. J., eds. Academic Press, New York, N. Y.
Prestwich, G. D. and Blomquist, G. B. 1987. "Pheromone Biochemistry." Academic Press,New York, N.Y. 565 pp.
Raina, A. K. and Menn, J. J. 1987. Endocrine regulation of pheromone production in Lepidoptera. pp 159-174. in: "Pheromone Biochemistry." Prestwich, G. D. and Blomquist, G. J., eds. Academic Press, New York, N.Y.
Rogoff, W. M., Beltz, A. D., Johnsen, J. O. and Plapp, F. W. 1964. A sex pheromone in the housefly, *Musca domestica*. J. Insect Physiol. 101: 239-246.
Rogoff, W. M., Gretz, G. H., Sonnet, P. F. and Schwarz, M. 1980. Response of male houseflies to muscalure and to combinations of hydrocarbons with and without muscalure. Environ. Entomol. 9: 605-606.
Roth, L. M., and Willis, E. R. 1952. A study of cockroach behavior. Am. Midl. Nat. 47: 65-129.
Roth, L. M., and Barth, R. J., Jr. 1964. The control of sexual receptivity in female cockroaches. J. Insect Physiol. 10: 965-975.
Schal, C. 1988. Regulation of pheromone synthesis and release in cockroaches. pp 695-700 in: "Endocrinological Frontiers in Physiological Insect Ecology." Sehnal, F., Zabza, A., and Denlinger, D.L., eds. Wroclaw Tech. Univ. Press, Wroclaw, Poland.
Schal, C., Burns, E. L., and Blomquist, G. J. 1990a. Endocrine regulation of female contact sex pheromone production in the German cockroach, *Blattella germanica*. Physiol. Entomol. 15: 81-91.
Schal, C., Burns, E. L., Jurenka, R. A., and Blomquist, G. J. 1990b. A new component of the female sex pheromone of *Blattella germanica*, and interaction with other pheromone components. J. Chem. Ecol. 16: 1997-2008.
Schal, C., and Smith, A. F. 1989. Neuroendocrine regulation of pheromone production in cockroaches. pp 179-200 in: "Cockroaches as models for neurobiology: Applications in biomedical research." Huber, I., Rao, B.R., and Masler, E.P., eds. CRC Press, Boca Raton, Florida.
Schlein, T, Galun, R. and Ben-Eliahu, M. N. 1980. The legs of *Musca domestica* and *Glossina morsitans* females as the site of sex pheromone release. Experientia 36: 1174-1175.
Smith, A. F., and Schal, C. 1990a. Corpus allatum control of sex pheromone production and calling in the female brown-banded cockroach, *Supella longipalpa*. J. Insect Physiol. (in press).
Smith, A. F., and Schal, C. 1990b. The physiological basis for the termination of pheromone-releasing behaviour in the female brown- banded cockroach, *Supella longipalpa*. J. Insect Physiol. (in press).
Smith, A. F., Yagi, K., Tobe, S. S. and Schal, C. 1989. *In vitro* juvenile hormone biosynthesis in adult virgin and mated female brown-banded cockroaches, *Supella longipalpa*. J. Insect Physiol. 35: 781-785.
Stay, B., Tobe, S. S., Mundall, E. C., and Rankin, S. 1983. Ovarian stimulation of juvenile hormone biosynthesis in the viviparous cockroach, *Diploptera punctata*. Gen. Comp. Endocr. 52: 341-349.
Tamaki, Y. 1985. Sex pheromones. Vol. 9 pp 145-192 in: "Comprehensive Insect Physiology, Biochemistry, and Pharmacology." G.A. Kerkut and L.I. Gilbert, eds. Pergamon Press, Oxford.
Tang, J. D., Charlton, R. E., Jurenka, R. A., Wolf, W. A., Phelan, P. L., Sreng, L. and Roelofs, W. L. 1989. Regulation of pheromone biosynthesis by a brain hormone in two moth species. Proc. Natl. Acad. Sci. USA 86: 1806-1810.
Uebel, E. C., Schwarz, M., Lusby, W. R., Miller, R. W. and Sonnet, P. E. 1978. Cuticular non-hydrocarbons of the female housefly and their evaluation as mating stimulants. Lloydia 41: 63-67.
Uebel, E. C., Sonnet, P. E. and Miller, R. W. 1976. Housefly sex pheromone: Enhancement of mating strike activity by combination of (Z)-9-tricosene with branched saturated hydrocarbons. J. Econ. Entomol. 5: 905-908.
Vanderwel, D. and Oehlschlager, A. C. 1987. Biosynthesis of pheromones and endocrine regulation of pheromone production in Coleoptera. pp 175-215 in: "Pheromone Biochemistry", Prestwich, G. D. and Blomquist, G. J., eds. Academic Press, New York, N.Y.

Vaz, A. H., Blomquist, G. J. and Reitz, R. C. 1988. Characterization of the fatty acyl elongation reactions involved in hydrocarbon biosynthesis in the housefly, *Musca domestica*. Insect Biochem. 18: 177-184.
Vaz, A. H., Brownson, E. A., Blomquist, G. J. and Reitz, R. C. 1989. Sex pheromone biosynthesis in the housefly: Evidence for the regulation of the fatty acyl-CoA desaturation and elongation system by 20-hydroxyecdysone. Arch Insect Biochem. Physiol. 12: 173-186-186.
Weaver, R. J. 1984. Effects of food and water availability, and of NCA-1 section, upon juvenile hormone biosynthesis and oocyte development in adult female *Periplaneta americana*. J. Insect Physiol. 30: 831-838.
Woodhead, A. P., and Stay, B. 1989. Neural inhibition of corpora allata in protein-deprived *Diploptera punctata*. J. Insect Physiol. 35: 415-421.

ENDOGENOUS REGULATION OF PHEROMONE BIOSYNTHESIS IN *Heliothis* MOTHS

James H. Tumlinson and Peter E. A. Teal

USDA-ARS
Insect Attractants, Behavior, and Basic
Biology Research Laboratory
Gainesville, Florida 32604

INTRODUCTION

Regulation of the diel periodic biosynthesis and release of sex pheromones by females of Lepidoptera is critical for reproductive success. Although other mechanisms often function in conjunction with temporally distinct pheromone communication periods to effect reproductive isolation (Sanders, 1971; Kaae et al., 1973; Roelofs and Cardé, 1974; Tamaki and Honma, 1976; Cardé et al., 1977; Grant, 1977), some cases have been documented in which differences in periodicity function as the major factor in reproductive isolation. Among noctuid moths, the prime importance of temporal regulation for maintenance of species isolation is exemplified by three, interfertile, sibling species of the genus Euxoa. Females of these moths produce a pheromone which is equally attractive to males of all three species (Byers et al., 1981). However, all three species have discrete reproductive periods which are tightly regulated so as to afford species specific, distinct peaks of reproductive activity under various temperature regimes (Teal et al., 1978; Teal and Byers, 1980).

Endogenous regulation of physiology and behavior associated with pheromone production results from the action of nervous, neuroendocrine and/or endocrine stimulation (Barth and Lester, 1973; Blomquist and Dillwith, 1983; Raina and Menn, 1987; Vanderwel and Oehlschlager, 1987). There are many facets of pheromone-mediated biology including initiation of biosynthesis, termination of production, actual release of compounds, and associated behavioral and physiological responses of the receiving sex. Although these processes are tightly coordinated, it is becoming obvious that no single neural, neuroendocrine or endocrine factor regulates the complete system.

INDUCTION OF PHEROMONE BIOSYNTHESIS

Studies conducted on cockroaches (Barth, 1961), which indicated that allatectomy inhibited the production of sex pheromone, led Barth (1965) to hypothesize that insects which are long-lived as adults, have a number of discrete reproductive cycles, and/or which have periods during which mating is not appropriate would regulate the production of sex pheromones by endocrine/neuroendocrine control. This hypothesis appears to be well founded because either the corpora allata, corpora cardiaca or ovaries regulate pheromone production in a number of species of Orthoptera, Coleoptera and the house fly, *Musca domestica,* (Barth and Lester, 1973; Blomquist and Dillwith, 1983; Adams et al., 1984). In the majority of these cases juvenile hormone (JH) has been shown to be involved directly with induction of biosynthesis (Barth and Lester, 1973; Blomquist and Dillwith, 1983; Vanderwel and Oehlschlager, 1987). However, ecdysone regulates pheromone biosynthesis in the house fly (Adams et al., 1984; Blomquist et al., 1987).

Molecular Insect Science, Edited by
H. H. Hagedorn *et al.,* Plenum Press, New York, 1990

Barth (1965) also hypothesized that neuroendocrine control of pheromone production would not occur in insects which are short-lived and reproductively mature at adult eclosion, citing results from studies on *Galleria mellonella* (Roller et al., 1963) and *Antheraea pernyi* (Barth, 1965), which indicated that allatectomy had no effect on pheromone production. However, extirpation of the corpora allata-corpora cardiaca complex of pupae of *Hyalophora cecropia* and *A. polyphemus* rendered the adult females unattractive to males (Riddiford and Williams, 1971). Although males did not respond, no quantitative data were given regarding the titer of pheromone present in the glands. Thus, pheromone could have been produced, but not released.

Hollander and Yin (1982, 1985) found that pheromone release was affected when the brain of the gypsy moth, *Lymantria dispar*, was removed, the suboesophageal connectives were severed, or when the abdominal nerve cord was severed. Extirpation of the corpora cardiaca and corpora allata had no effect on the release of pheromone. Consequently, these workers considered that neural input, rather than a humoral factor, regulated pheromone release. The hypothesis that the corpora allata does not influence pheromone release was supported in a study (Tang et al., 1987) which compared the amount of disparlure released by allatectomized, sham operated, and control females. This study also indicated that transection of the abdominal nerve cord of pupae anterior to the terminal abdominal ganglion (TAG) resulted in significant decreases in both the amounts of disparlure released and present in the gland. However, because pheromone production was not eliminated when the abdominal ganglion was severed, these authors suggested that both humoral and neural factors regulated pheromone production.

Pheromone Biosynthesis Activation in Moths

Raina and Klun (1984) demonstrated that females of *Heliothis zea*, ligated between the head and thorax, did not produce pheromone during the calling period. They showed that injection of homogenates of the brains and suboesophageal ganglia of females into the abdomens of ligated females resulted in production of pheromone titers which were equivalent to those produced by untreated calling females. Their study also indicated that extracts of the brains of male insects could induce pheromone production, as could extracts obtained from females during the period when pheromone titer was lowest. They islolated a compound, subsequently shown to be a neuropeptide (Jaffe et al., 1986; Raina et al., 1987), that induced pheromone biosynthesis. Detailed studies have shown that the neuropeptide, termed pheromone biosynthesis activating neuropeptide (PBAN), is present in the suboesophageal ganglion (SOG) (Raina et al., 1987). Based on the fact that a small amount of pheromonotropic activity was associated with extracts of the corpora cardiaca and with hemolymph obtained from calling females and on unpublished results (cited by Raina and Menn, 1987; and Raina et al., 1987), Raina and Menn (1987) have proposed that PBAN, synthesized in the SOG, is carried to the corpora cardiaca, released into the hemolymph and is transported to the pheromone gland where it acts to induce biosynthesis. They suggested that the action of PBAN is promoted during the dark period and inhibited during the photophase by light-activated enzymes which degrade PBAN and metabolize any pheromone produced.

Experiments conducted in our laboratory on *H. zea, H. virescens, H. subflexa, Plodia interpunctella, Manduca sexta*, and *Spodoptera frugiperda* confirmed that PBAN is produced by the SOG and that it does induce pheromone biosynthesis, as have recent reports by Ohguchi et al. (1985) and Ando et al. (1988). However, we found that females of the *Heliothis* species could be induced to produce pheromone during the photophase by injection of homogenates of the SOG (hereafter termed crude PBAN) (Teal and Tumlinson, 1989). The pheromone blend produced by females of *H. zea* consists of four aldehydes as indicated in Figure 1. The (Z)-11-hexadecenol (Z11-16:OH) shown in the chromatogram is the precursor of the major component of the pheromone, (Z)-11-hexadecenal (Z11-16:AL) (Teal and Tumlinson, 1986). Normally the amount of pheromone present in the gland during the photophase is very low (less than 1 ng of the major component, Z11-16:AL), compared with that found during the calling period of the scotophase (ca. 60 μg of Z11-16:AL). When intact females were injected with crude PBAN during the photophase they produced amounts of pheromone and ratios of components that were equivalent to neck ligated insects injected with crude PBAN during the scotophase (Teal and Tumlinson, 1989).

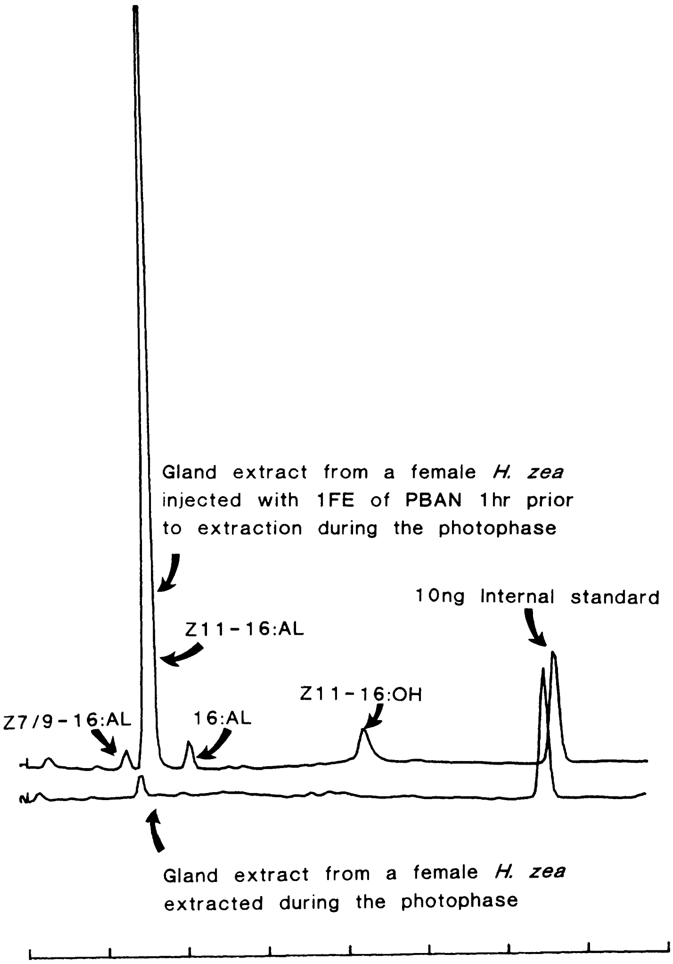


Fig. 1. Gas chromatographic analysis (column = 30 m x 0.25 mm id SPB1®) of the extract of a pheromone gland from a 3-day-old virgin female excised and extracted 1 hr after injection of 1 female equivalent (FE) of PBAN during the photophase (upper) compared with the extract of a pheromone gland obtained 1 hr after injection of buffer during the photophase. Compounds present include (Z)-7-hexadecenal and (Z)-9-hexadecenal (Z7/9-16:AL) (no resolution), (Z)-11-hexadecenal (Z11-16:AL),, hexadecanal (16:AL) and (Z)-11-hexadecen-1-ol (Z11-16:OH), the precursor of Z11-16:AL.

Furthermore, decapitated females and isolated abdomens also produced pheromone when injected with the crude homogenate during the photophase. Our data showed that light-activated enzymes do not degrade PBAN nor does enzymatic degradation of pheromone occur at a greater rate during the photophase.

Site of action of PBAN

We have found that females injected with 1 female equivalent (FE) of PBAN during the photophase produce, in 1 hr, an amount of pheromone equal to that present in the glands of calling females at the peak of pheromone production. However, when we incubated isolated pheromone glands with PBAN *in vitro* an increase in pheromone production did not occur (Fig. 2) (Teal et al., 1989). We discovered that this is because PBAN does not act directly on the cells of the pheromone gland, but on the TAG. Severing the ventral nerve cord anterior to the TAG prior to injection of PBAN did not inhibit pheromone production

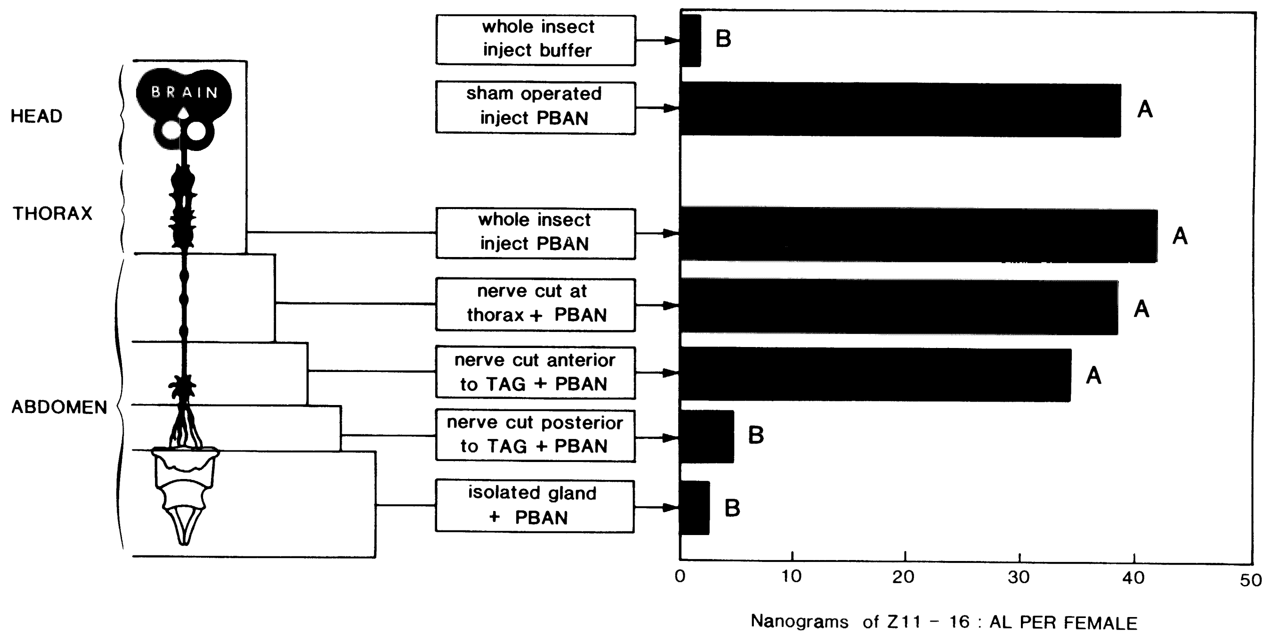


Fig. 2. Amount of Z11-16:AL produced by the pheromone gland in the 1-hr period after injection of 1 female equivalent of PBAN into intact *H. zea* females; females in which the abdominal nerve cord was cut anterior or posterior to the terminal abdominal ganglion; or sham-operated females; and on *in vitro* incubations of gland plus PBAN. All experiments were conducted during the third or fourth photophase after adult emergence and were replicated 10 times. Means were analyzed by a Duncan's multiple range test. Bars capped with the same letter are not significantly different ($P = 0.05$).

(Fig. 2). However, when nerves extending from the TAG into the area of the pheromone gland were severed prior to PBAN injection no significant level of pheromone was produced (Fig. 2). When the TAG was bathed in PBAN while still attached to the gland so that no PBANcontacted the gland, pheromone was produced. Severing the nerves extending to the gland inhibited pheromone production in these preparations. Thus, PBAN stimulates the TAG which then sends a second message to the pheromone gland. Synaptic connections with individual gland cells have never been found in histological preparations (Teal et al., 1983; Aubrey et al., 1983), but several cell somata have been found in the TAG when nerves extending into the immediate vicinity of the pheromone gland were backfilled with cobalt lysine (Itagaki, pers. comm.). This suggests that the second message is neuron-mediated.

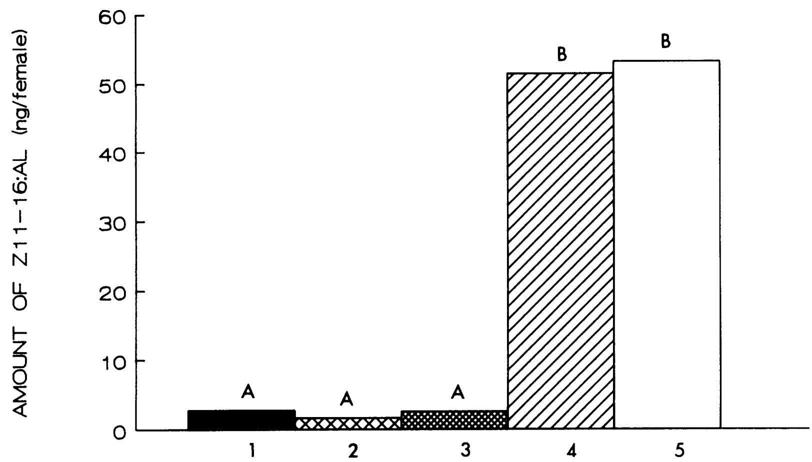


Fig. 3. Amount of Z11-16:AL present in glands of 3-day-old virgin *H. zea* females under the following conditions: 1-during the light period (solid bar); 2-abdominal nerve cord severed anterior to the terminal abdominal ganglion during the day but sampled at the peak of pheromone production of the subsequent night (cross-hatched bar); 3-abdominal nerve cord severed anterior to the first abdominal ganglion and treated as in 2 (small cross-hatched bar); 4-abdominal nerve cord severed as in 2 but injected with 1 female equivalent of PBAN 1 hr prior to extraction during the subseqauent night (hatched bar); 5-during the peak of pheromone production in untreated females (open bar). Bars capped with the same letter are not significantly different in a Duncan's multiple range test (P = 0.05) (n = 10 for each treatment).

Transmission of PBAN to the terminal abdominal ganglion

Raina and Klun (1984) found a small, but significant amount of pheromonotropic activity associated with the hemolymph. We tested the hypothesis that PBAN is carried to the TAG via the hemolymph by injecting blood obtained from 2-4 calling females into females which were not producing pheromone. In more than 30 trials no females injected with hemolymph produced more pheromone than control females injected with buffer (Teal et al., 1989). However, we found that connection between the TAG and the more anterior ganglia of the ventral nerve cord is required for induction of pheromone biosynthesis unless PBAN is injected into the insect (Teal et al., 1989). When we severed the abdominal nerve cord immediately anterior to the TAG or at the junction of the thorax during the light period and analyzed for the presence of pheromone 12 h later, when pheromone production should have been at its peak, we found only a small amount of pheromone (Fig. 3). However, when nerve transected insects were injected with PBAN they produced pheromone (Fig. 3).

Experiments conducted in our laboratory have shown that pheromonotropic activity is associated with the abdominal nerve cord of calling insects. In this study we removed and homogenized the entire abdominal nerve cord or portions of it from calling females and then injected this homogenate into the abdomens of non-calling insects during the light cycle. The greatest activity is associated with either the entire abdominal nerve cord or the three ganglia and connectives anterior to the TAG (Fig. 4) (see Chauthani and Callahan, 1967).

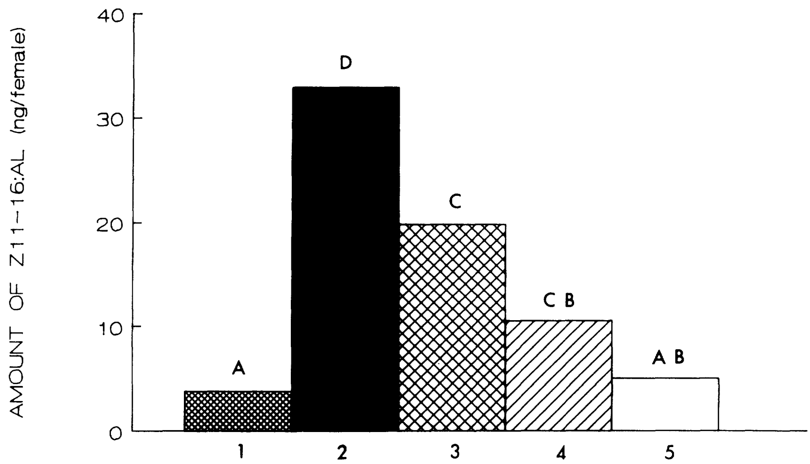


Fig. 4. Production of Z11-16:AL by intact virgin *H. zea* females during the third photophase after emergence following injection of the following: 1-10 µl of buffer (small cross-hatched bar) n = 10); 2-homogenates of the entire abdominal nerve cord obtained from calling females (solid bar) (n = 12); 3-homogenates of the three ganglia and connectives anterior to the terminal abdominal ganglion from calling females (cross-hatched bar) (n = 10); 4-homogenates of the terminal abdominal ganglia obtained from females during the peak of pheromone production (hatched bar) n = 10); 5-effect of injection of 2 female equivalents of homogenates of the entire abdominal ganglia obtained from females during the period in which the pheromone titer is lowest (n = 10). Bars capped with the same letter are not significantly different in a Duncan's multiple range test (p = 0.05).

Homogenates of the abdominal nerve cords taken from females that were not producing pheromone had only limited activity. The lower activity associated with the TAG could be the result of the smaller size of the TAG relative to the remaining abdominal nerve cord, or metabolism of this pheromonotropic factor by the TAG. Thus the abdominal nerve cord appears to be the vehicle by which PBAN reaches the TAG.

TERMINATION OF PHEROMONE BIOSYNTHESIS

After copulation, female insects usually enter a period during which they deposit fertilized eggs and are not engaged in production of the sex pheromone (Barth and Lester, 1973). This switch from “virgin" to “mated" behavior is transitory in species which mate more than once. The behavioral change associated with mating can be the result of mechanical stimulation, as occurs in a number of Orthoptera (Loher and Huber, 1966; Roth, 1962; 1964), or it can be chemically induced (Barth and Lester, 1973).

In *M. domestica*, the males release a chemical from ejaculatory ducts during mating which is responsible for inducing the “mated" condition in females (Riemann et al., 1967). This mating inhibitor is absorbed into the hemolymph of the female, through the lining of the vaginal pouches, and is carried to the brain where it is thought to bind with specific neural receptors (Leopold and Terranova, 1970; Leopold et al., 1971). Similarly, males of *Aedes aegypti* produce a substance, matrone, which results in inhibition of sexual receptivity in inseminated females (Craig, 1967). Interestingly, matrone appears to act on the TAG to inhibit further sexual behavior (Gwadz, 1972).

The first evidence for endocrine regulation of the shift from "virgin" to"“mated" behavior for Lepidoptera was provided by Truman and Riddiford (1971) and Riddiford and Ashenhurst (1973). Their studies demonstrated that after a fertile mating, females of *H. cecropia* release "bursa factor" into the hemolymph. This appears to interact with cells of the corpora cardiaca to stimulate the release of an oviposition hormone. It is of interest to note that the presence of sperm is critical for the release of "bursa factor", because such a system allows females to remain sexually active after an infertile mating. It is unknown if

the "bursa factor" also exerts some inhibitory action on the biosynthesis of sex pheromone.

Webster and Cardé (1984) provided evidence for hormone-induced inhibition of pheromone production by females of the omnivorous leafroller moth. Their study indicated that application of juvenile hormones or hydroprene inhibited calling and stimulated oviposition. This suggested that juvenile hormone might be involved in the switch from the virgin to mated condition. Chang et al. (1979) found that precocene II had a similar effect on virgin females of the diamondback moth, in as much as treated females were less attractive to males than untreated insects.

Recently Raina (1988) has provided evidence that naturally produced chemicals inhibit pheromone biosynthesis in moths. In earlier studies Raina et al. (1986) showed that pheromone titer dropped substantially almost immediately after mating, and that the titer of pheromone remained lower in mated females than that present in virgins for three days following mating. Raina (1988) found that when hemolymph, obtained from females 10 min after mating, was injected into virgins, pheromone biosynthesis was inhibited and pheromone titer dropped significantly. This suggested that this factor not only inhibited biosynthesis but also increased the metabolism of pheromone. The inhibitory activity was also associated with the hemolymph of males and was unaffected by castration of males during the larval stage. Thus, Raina (1988) hypothesized that a factor, perhaps produced in the male accessory glands, is transferred with the spermatophore at mating. He suggests that this factor is then carried to the brain in a fashion similar to that outlined for the house fly (Leopold et al., 1971).

Suppression of Pheromone Biosynthesis in Virgin Female Moths

Unlike females of *H. cecropia*, virgin females of *H. zea* begin to oviposit at a significant rate on the fourth night of adult life and produce very little sex pheromone thereafter compared to younger adults. Since the amount of pheromone produced after injection of PBAN into 3-day-old adults increases with increasing doses of the neuropeptide we hypothesized that the reduction in pheromone titer in aging virgin females was due to the production of smaller amounts of PBAN. To test this hypothesis we injected females during the 3rd photophase after emergence with 1 female equivalent of crude PBAN obtained from donor insects which were 5 days old. Surprisingly, the injected insects produced an average of 55.3 ng (±13.0, SD, n=5) of Z11-16:AL, statistically equivalent to the 53.6 ng (±16.4, SD) produced when the insects were injected with 1 female equivalent of crude PBAN obtained from 3-day-old females (Teal et. al., 1990). Furthermore, when we injected crude PBAN obtained from 3-day-old females into 5-day-old females they produced significantly less pheromone (17.0 ng, ±7.8, SD). Thus, although PBAN obtained from both groups of females was equally active, 5-day-old females were unable to produce as much pheromone as females that were at their reproductive peak. Other studies (Teal and Tumlinson, 1986) have shown that the enzymes responsible for the terminal steps in pheromone biosynthesis are as active in 5-day-old females as they are in 3-day-old insects. Furthermore, studies have shown that substrate availability, rather than activation of specific enzymes involved in pheromone biosynthesis, regulates the production of pheromones in moths (Tang et al., 1989; Teal and Tumlinson, 1986). These observations have led us to conclude that the reduction in pheromone titer associated with increasing age was not due to either the rate of production and release of PBAN, or the biochemical readiness of the pheromone gland to synthesize pheromone. Therefore, it appears that a second factor other than PBAN is affecting pheromone production in the older virgin females.

Source of Pheromone Biosynthesis Suppression Factor

Knowing that the bursa copulatrix of mated female *H. cecropia* releases a substance that causes a shift from virgin to mated behavior (Riddiford and Ashenhurst, 1973) and that secretory cells are present in the bursa copulatrix of Lepidoptera (Klatt, 1920; Omura, 1938; Callahan and Cascio, 1963), we hypothesized that some hormonal factor responsible for suppression of pheromone biosynthesis might be produced by the bursa copulatrix. We demonstrated that this was, in fact, true because when we injected 2 female equivalents of the aqueous homogenate of the bursa copulatrix obtained from virgin 5-day-old females along with 1 female equivalent of crude PBAN, the amount of pheromone present in the gland 1 hr later was significantly lower than that present in glands of females injected with crude PBAN alone in an equivalent amount of buffer (Table 1). Injection of 2 female equi-

valents of the homogenate of either the cervix bursae or corpus bursae (see Callahan, 1958) along with 1 female equivalent of crude PBAN also resulted in significant reductions in the amount of pheromone produced (Table 1). No decrease in pheromone titer was noted when extracts of the bursa copulatrix obtained from females during the first photophase after adult emergence was tested. However, insects injected with 1 female equivalent of crude PBAN plus 2 female equivalent of the bursa copulatrix extract obtained from females during the third photophase produced less than 50% of the amount of pheromone produced by females which had been injected with crude PBAN alone. Thus, the titer of the suppression factor present in the bursa copulatrix increases with increasing age and appears to be correlated with both increased oviposition and decreased pheromone production.

Table 1. Effect of injection of PBAN plus 2 female equivalents of tissue extracts obtained from 5-day-old virgin females on pheromone production* in 3-day-old virgin females of *H. zea*.

TREATMENT	% PHEROMONE	
1 FE PBAN + 20 µl buffer (n = 10)	100.0	A#
1 FE PBAN + 2 FE unwashed bursa copulatrix (n = 10)	2.9	E
1 FE PBAN + 2 FE washed bursa copulatris (n = 5)	5.1	E
1 FE PBAN + 2 FE corpus bursa (n = 5)	32.9	BC
1 FE PBAN + 2 FE cervix bursa (n = 5)	57.0	B
1 FE PBAN + 2 FE washed ovary (n = 5)	12.4	D
1 FE PBAN + 2 FE washed fat body (n = 5)	91.3	A
1 FE PBAN + 2 FE washed thoracic muscle (n = 5)	104.7	A
1 FE PBAN + 2 FE unwashed thoracic muscle (n = 5)	32.5	BC
1 FE PBAN + 2 FE hemolymph (n = 5)	25.3	C

* Pheromone production as indicated by the titer of Z11-16:AL in gland extracts.

Percentages followed by the same letter are notsignificantly different as indicated by a Duncan's multiple range test at p = 0.05 using the amount (ng) of Z11-16:AL present in extracts.

We conducted concentration gradient studies using various amounts of the extract of the bursa copulatrix and found that the suppressive effect increases in a linear fashion with increasing doses up to 2 female equivalents (Fig. 5). Complete suppression of pheromone production resulted when 2 female equivalents of the extract were administered along with 1 female equivalent of crude PBAN.

To determine if the suppression of pheromone biosynthesis was caused by a hormone obtained from the bursa copulatrix, rather than just a nonspecific response to the injection of tissue extracts, we injected homogenates of thoracic muscle, abdominal fat body, and ovaries. Although neither homogenates of the washed fat body nor washed thoracic muscle inhibited the pheromonotropic effect of crude PBAN injected concomitantly, injection of homogenates of the ovaries resulted in a significant reduction in pheromone (Table 1). However, this reduction was not as great as that which occurred when extracts of the bursa copulatrix were administered along with crude PBAN. Therefore, the suppressive effect is specifically associated with the bursa copulatrix and ovaries, two closely associated and interconnected organs. Interestingly, although homogenates of the washed thoracic muscle did not contain the suppression factor, injection of crude PBAN plus homogenates of the thoracic tissue prepared without prewashing resulted in the production of significantly less pheromone than that produced by insects treated with crude PBAN alone (Table 1). This suggested that the suppressive substance was present in the hemolymph of older females as would be expected of a hormone. When hemolymph obtained from older females was injected along with crude PBAN a decrease in pheromone production was noted, but the reduction was not as great as that resulting from injection of extracts of the bursa copulatrix. This may be accounted for by the small volume of hemolymph obtained from these insects relative to the total blood volume. Nonetheless, hemolymph does contain the suppression factor.

Knowing that juvenile hormones affect pheromone production in several species of insects (Barth and Lester, 1973; Vanderwell and Oehlschlager, 1987; Cusson and McNeil, 1989) and that topical application of a juvenile hormone analog hydroprene caused females of the omnivorous leafroller moth to oviposit while inhibiting both calling behavior and pheromone production (Webster and Cardé, 1984), we hypothesized that juvenile hormone

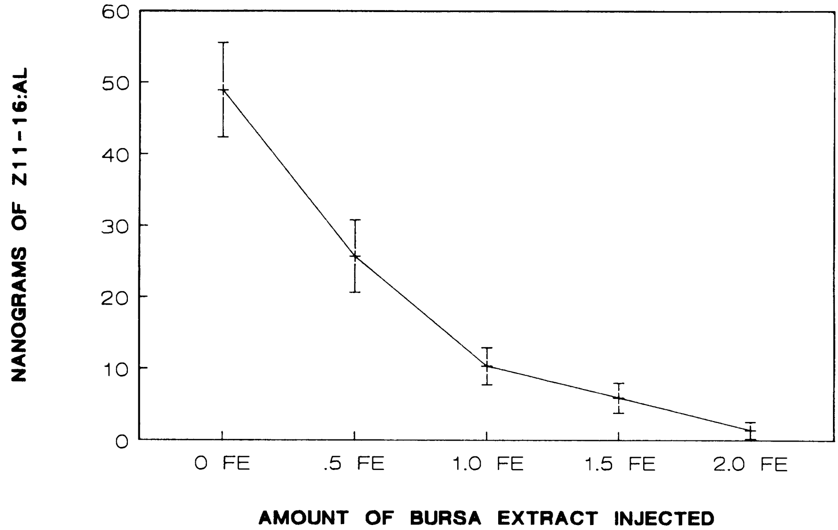


Fig. 5. Amount of Z11-16:AL produced by intact 3-day-old virgin *H. zea* females 1 hr after injection of different concentrations of the extract of the bursa copulatrix obtained from 5-day-old virgin females together with 1 female equivalent of PBAN obtained from 3-day-old females (n = 10, each concentration, ± SD).

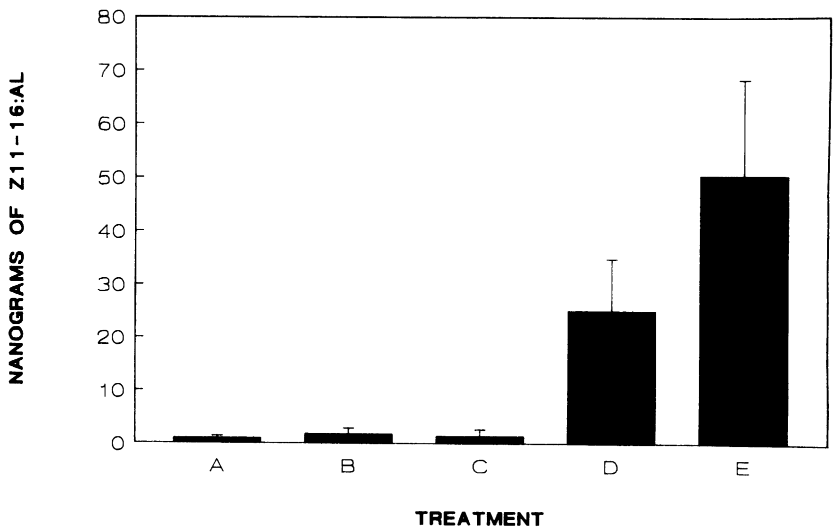


Fig. 6. Production of Z11-16:AL by intact virgin *H. zea* females injected during the third photophase after emergence with 2 female equivalents of the extract of the bursa copulatrix obtained from 5-day-old virgin females and/or 1 female equivalent of PBAN from 3-day-old females. Pheromone glands were extracted 60 min after injection of PBAN (n = 5, each treament, ± SD). A-injection of bursa extract 60 min prior to PBAN; B-injection of bursa extract 30 min prior to PBAN; C-injection of bursa extract at the same time as PBAN (mixed together); D-injection of bursa extract 30 min after PBAN; E-injection of PBAN alone.

might be the factor responsible for suppressing pheromone biosynthesis. Injection of 50 ng of juvenile hormone I, II or III along with crude PBAN resulted in 3-4 fold reductions in the titer of Z11-16:AL relative to control insects injected with only crude PBAN. However, extraction of the homogenates of both the bursa copulatrix and ovaries with pentane, hexane, or iso-octane, solvents known to extract juvenile hormones from aqueous homogenates (Schooley, 1977; Feyereisen and Tobe, 1981), did not alter the suppressive activity of the aqueous phase nor was any activity associated with the lipids extracted into the organic solvents. Gas chromatographic analysis of the lipids extracted by the organic solvents did not reveal the presence of juvenile hormones I, II, or III. Therefore, although juvenile hormones mimic the effect of the suppression factor when administered at levels presumed to be physiologically high, the substance present in the bursa copulatrix and ovaries is not one of the juvenile hormones. Similar experiments using doses from 10-400 ng of either ecdysone or 20-hydroxy ecdysone mixed with 1 female equivalent of crude PBAN did not result in the production of less pheromone than that produced by control insects injected with crude PBAN alone. Thus, the pheromone biosynthesis suppression factor is different from the known insect hormones.

Interaction of PBAN and PBSF

We have found that injection of the bursa copulatrix extract both 30 min and 60 min prior to injection of PBAN completely suppresses pheromone production (Fig. 6). Moreover, injection of this extract 30 min after injection of crude PBAN stops pheromone production immediately but does not cause increased metabolism of the pheromone already produced due to PBAN stimulation. Thus, the glands of females injected with the extract of the bursa copulatrix 30 min. after injection of crude PBAN contained an average of 25.1 ± 6.8 ng (n = 5) of Z11-16:AL when extracted 30 min later, whereas females injected with crude PBAN only 30 min prior to extraction of the glands contained an average of 21.6 ± 5.6 ng (n=5). Consequently, it appears that the factor present in the bursa copulatrix acts to suppress the action of PBAN instantaneously. We are in the process of isolating and identifying this factor and are currently studying the mechanism by which it suppresses pheromone biosynthesis.

Pheromone biosynthesis suppression factor

The fact that virgins produce this hormone, which we term "pheromone biosynthesis suppression factor" (PBSF), as they age is somewhat factitious in as much as, in nature, the vast majority of females mate at least once, and it is well established that mated females produce less pheromone than their virgin counterparts. Therefore, we believe that under natural field conditions PBSF may be released in response to placement of the spermatophore in the bursa copulatrix during mating, and that such release of PBSF results in the much reduced pheromone titer present in the glands of mated females, thereby rendering them unattractive to males. Hormonal regulation of this type by moth species is advantageous to the species because it reduces competition between mated and virgin females, thus increasing the probability that virgins will mate and thereby increasing genetic diversity. In addition, the refractory period after mating, when pheromone titer is very low, would allow females to lay fertilized eggs without threat of being accosted by sexually aroused males.

SUMMARY

Although the chemistry and biological effects of sex pheromones are understood more fully for moth species than for any other group of insects, the endogenous mechanisms which regulate the initiation and termination of pheromone biosynthesis remain largely unexplored. These neural, neurohormonal and hormonal regulators effectively control the periodicity of sexual signaling and are thus of prime importance for the reproductive success of moth species. Recent findings in our studies on *Heliothis* moths have shown that pheromone biosynthesis activating neuropeptide (PBAN) produced by the suboesophageal ganglion does not act on the pheromone gland, but, rather, on the terminal abdominal ganglion (TAG) which innervates the pheromone gland area. This ganglion then sends a second message, different from PBAN, to the gland thus inducing pheromone production.

Additionally, a factor has been discovered that is produced by virgin females that causes rapid termination of pheromone biosynthesis. While these results begin to explain how pheromone production is controlled, they also raise several questions that need to be addressed.

REFERENCES

Adams, T. S., Dillwith, J. W., and Blomquist, G. J. 1984. The role of 20-hydroxy-ecdysone in housefly sex pheromone biosynthesis, J. Insect Physiol. 30: 287-294.

Ando, T., Arima, R., Uchiyama, M., Nagasawa, H., Inoue, T. and Suzuki, A. 1988. Pheromone biosynthesis activating neuropeptide hormone in heads of the silkworm moth, Agric. Biol. Chem. 52: 881-883.

Aubrey, J. G., Boudreaux, H. B., Grodner, M. L., Hammond, A. M. 1983. Sex pheromone-producing cells and their associated cuticle in female *Heliothis zea* and *H. virescens*, *Ann. Entomol. Soc. Am.*, 76: 343-348.

Barth, R. H., 1961, Hormonal control of sex attractant production in the Cuban cockroach, Science, 133: 1598-1599.

Barth, R. H., 1965, Insect mating behavior: Endocrine control of a chemical communication system, Science, 149: 882-883.

Barth, R. H. and Lester, L. J. 1973. Neuro-hormonal control of sexual behavior in insects, Annu. Rev. Entomol., 18: 445-472.

Blomquist, G. J. and Dillwith, J. W. 1983 Pheromones: Biochemistry and physiology. pp. 527-542 in: "Endocrinology of Insects." Vol. 1. Downer, R. G.and Laufer, H. eds. Alan R. Liss, New York.

Blomquist, G. J., Dillwith, J. W., and Adams, T. S. 1987 Biosynthesis and endocrine regulation of sex pheromone production in Diptera. pp. 217-250 in: "Pheromone Biochemistry." Prestwich, G. D. and Blomquist, G. J., eds. Academic Press, Orlando.

Byers, J. R., Underhill, E. W. Steck, W. F., Chisholm, M. D., and Teal, P. E. A. 1981. Biosystematics of the genus *Euxoa*. XV. Sex pheromone cross-attraction among three closely related species of the *declarata* group. Can. Entomol. 113: 235-243.

Callahan, P. S., 1958. Serial morphology as a technique for determination of reproductive patterns in the corn earworm, *Heliothis zea.* Ann. Entomol. Soc. Am. 51 :413.

Callahan, P. S. and Cascio, T. 1963 Histology of the reproductive tracts and transmission of sperm in the corn earworm, *Heliothis zea*. Ann. Entomol. Soc. Am., 56: 535-556.

Cardé, R. T., Cardé, A. M. Hill, A. S., and Roelofs, W. L. 1977. Sex pheromone specificity as a reproductive isolating mechanism among the sibling species *Archips argyrospilus* and *A. mortuanus* and other sympatric tortricine moths. J. Chem. Ecol. 3: 71-84.

Chang, F., Kou, R. and Chow, Y. S.. 1979. The effect of precocene II on sex attractancy in the diamondback moth, *Plutella xylostella*. Proc. Natl. Sci. Council Rep. China, 3 67-76.

Chauthani, A. R. and Callahan, P. S. 1967. The nervous system of the corn earworm moth, *Heliothis zea*. Ann. Entomol. Soc. Am. 60: 248-255.

Craig, G. B. 1967. Mosquitoes: Female monogamy induced by male accessory gland substance. Science 156: 1499-1501.

Cusson, M. and McNeil, J. N. 1989 Involvement of juvenile hormone in the regulation of pheromone release activities in a moth. Science 243 :210-212.

Feyereisen, R. and Tobe, S. 1981. A rapid partition assay for routine analysis of juvenile hormone release by insect corpora allata. Analyt. Biochem. 111: 372-375.

Grant, G. G. 1977. Interspecific pheromone responses of tussock moths and some isolating mechanisms of eastern species. Environ. Entomol. 6: 739-742.

Gwadz, R. W. 1972. Neurohormonal regulation of sexual receptivity in female *Aedes aegypti*, J. Insect Physiol. 18: 259-266.

Hollander, A. L. and Yin, C. M. 1982. Neurological influences on pheromone release and calling behavior in the gypsy moth. *Lymantria dispar*. Physiol. Entomol. 7: 163-166.

Hollander, A. L. and Yin, C. M. 1985. Lack of humoral control in calling and pheromone release by brain, corpora cardiaca, corpora allata and ovaries of the female gypsy moth, *Lymantria dispar*. J. Insect Physiol. 31: 159-163.

Jaffe, H., Raina, A. K. and Hayes, D. K. 1986. HPLC isolation and purification of pheromone biosynthesis activating neuropeptide of *Heliothis zea*. pp. 219-224 in:

"Proc. Int. Conf. Insect Neurochem. Neurophysiol." Borkovec, A. B. and Gelman, D. B., eds. Humana Press. Clifton.
Kaae, R. S., Shorey, H. H., McFarland, S. U. and Gaston, L. K. 1973. Sex pheromones of Lepidoptera. XXXVII. Role of sex pheromones and other factors in reproductive isolation among ten species of Noctuidae. Ann. Entomol. Soc. Am. 66: 444-448.
Klatt, B. 1920. Beiträge zur Sexualphysiologie des Schwammspinners, Biol. Zentralb., 40: 539-558.
Leopold, R. A. and Terranova, A. C. 1970. The distribution and persistence of ^{3}H-labelled male accessory secretion in mated female houseflies. Proc. N. Dak. Acad. Sci. 24: 20-20.
Leopold, R. A., Terranova, A. C., and Swilley, E. M. 1971. Mating refusal in *Musca domestica*: Effects of repeated mating and decerebration upon frequency and duration of copulation. J. Exp. Zool. 176: 353-360.
Loher, W. and Huber, F. 1966. Nervous and endocrine control of sexual behavior in a grasshopper (*Gomphocerus rufus*). Symp. Soc. Exp. Biol. 20: 381-400.
Ohguchi, Y., Tatsuki, S., Usui, K., Arai, K., Kurihara, M., Uchiumi, K. and Fukami, J. 1985. Hormone-like substance present in the cephalic organs of the female moth, *Chilo suppressalis* and controlling sex pheromone production. Jap. J. Appl. Entomol. Res. 29: 265-269.
Omura, S. 1938. Structure and function of the female genital system of *Bombyx mori* with special reference to the mechanism of fertilization. J. Fac. Agric. Hokkaido Imp. Univ. 40: 111-128.
Raina, A. K. 1988. Selected factors influencing neurohormonal regulation of sex pheromone production in *Heliothis* species. *J. Chem. Ecol.* 14: 2063-2069.
Raina, A. K. and Klun, J. A. 1984. Brain factor control of sex pheromone production in the female corn earworm moth. Science 225: 531-533.
Raina, A. K. and Menn, J. J. 1987. Endocrine regulation of pheromone production in Lepidoptera. pp. 159-174 in: "Pheromone Biochemistry." Prestwich, G. D. and Blomquist, G. J., eds. Academic Press, Orlando.
Raina, A. K., Klun, J. A., and Stadelbacher, E. A. 1986. Diel periodicity and effect of age and mating on female sex pheromone titer in *Heliothis zea*. Ann. Entomol. Soc. Am. 79: 128-131.
Raina, A. K., Jaffe, H., Klun, J. A., Ridgway, R. L., and Hayes, D. K. 1987 Characteristics of a neurohormone that controls sex pheromone production in *Heliothis zea*. J. Insect. Physiol. 33: 809-814.
Riddiford, L. M. and Ashenhurst, J. B. 1973. The switchover from virgin to mated behavior in female cecropia moths: The role of the bursa copulatrix. Biol. Bull. 144: 162-171.
Riddiford, L. M. and Williams, C. M. 1971. Role of corpora cardiaca in the behavior of saturniid moths. I. Release of sex pheromone. Biol. Bull. 140: 1-7.
Riemann, J. G., Moen, D. J., and Thorson, B. J. 1967. Female monogamy and its control in houseflies. J. Insect. Physiol. 13: 407-418.
Roelofs, W. L. and Cardé, R. T. 1974. Sex pheromones in the reproductive isolation of lepidopterous species. pp. 96-114 in: "Pheromones." Birch, M. C., ed. North-Holland, Amsterdam.
Roller, H., Piepho, H., and Holz, I. 1963. The problem of hormone dependency of copulation behaviour in insects. Studies of *Galleria mellonella* J. Insect. Physiol. 9: 187-194.
Roth, L. M. 1962. Hypersexual activity induced in females of the cockroach *Nauphoeta cincerea*. Science 138: 1267-1269.
Roth, L. M.. 1964. Control of reproduction in female cockroaches with special reference to *Nauphoeta cinerea*. I. First pre-oviposition period. J. Insect. Physiol. 10: 915-945.
Sanders, C. J. 1971. Daily activity patterns and sex pheromone specificity as sexual isolating mechanisms in two species of *Choristoneura*. Can. Entomol. 103: 498-502.
Schooley, D. A. 1977. Analysis of the naturally occurring juvenile hormones - their isolation, identification and titer determination at physiological levels. pp 241-287 in: "Analytical Biochemistry of Insects." Turner, R. B., ed. Elsevier, New York.
Tamaki, Y. and Honma, K. 1976. Sex pheromone and reproductive isolation in Lepidoptera with special reference to *Adoxophyes* spp. in Japan. pp. 13-22 in: "Proceedings Symposium Insect Pheromones and Applications." Jap. Plant Protect. Assoc., Tokyo.
Tang, J. D., Charlton, R. E., Cardé, R. T., and Yin, C. M. 1987. Effect of allatectomy and ventral nerve cord transection on calling, pheromone emission and pheromone

production in *Lymantria dispar*. J. Insect Physiol. 33: 469-476.

Tang, J. D., Charlton, R. E., Jurenka, R. A., Wolf, W. A., Phelan, P. L., Sreng, L. and Roelofs, W. L. 1989. Regulation of pheromone biosynthesis in two moth species, Proc. Nat. Acad. Sci. 86: 1806-1810.

Teal, P. E. A. and Byers, J. R. 1980. Biosystematics of the genus *Euxoa*. XIV. Effect of temperature on female calling behavior and temporal partitioning of three sibling species of the declarata group. Can. Entomol. 112: 113-117.

Teal, P. E. A. and Tumlinson, J. H. 1986. Terminal steps in pheromone biosynthesis by *Heliothis virescens* and *H. zea*. J. Chem. Ecol. 12: 353-365.

Teal, P. E. A. and Tumlinson, J. H. 1989. Neurohormonal regulation of pheromone biosynthesis by *Heliothis zea* during the light period. Can. Entomol. 121: 43-46.

Teal, P. E. A., Byers, J. R. and Philogene, B. J. R. 1978. Differences in female calling behavior of three interfertile sibling species of *Euxoa*. Ann. Entomol. Soc. Am. 71: 630-634.

Teal, P. E. A., Carlysle, T. C. and Tumlinson, J. H. 1983. Epidermal glands in terminal abdominal segments of female *Heliothis virescens*. Ann. Entomol. Soc. Am. 76: 242-247.

Teal, P. E. A., Tumlinson, J. H., and Oberlander, H. 1989. Neural regulation of sex pheromone biosynthesis in *Heliothis* moths. Proc. Nat. Acad. Sci. 86: 2488-2492.

Teal, P.E.A., Tumlinson, J.H., and Oberlander, H. 1990. Endogenous suppression of pheromone production in virgin female moths. Experentia (in press).

Truman, J. W. and Riddiford, L. M. 1971. The role of the corpora cardiaca in the behavior of saturnid moths. II. Oviposition. Biol. Bull. 140: 8-14.

Vanderwel, D. and Oehlschlager, A. C. 1987. Biosynthesis of pheromones and endocrine regulation of pheromone production in Coleoptera. pp. 175-215 in: "Pheromone Biochemistry." Prestwich, G. D. and Blomquist, G. J., eds. Academic Press, New York.

Webster, R. P. and Cardé, R. T. 1984. The effect of mating, exogenous juvenile hormone and a juvenile hormone analogue on pheromone titer, calling and oviposition in the omnivorous leafroller moth (*Platynota stultana*). J. Insect Physiol. 30: 113-118.

CYTOCHROME P450 IN PLANT-INSECT INTERACTIONS: INDUCTIONS AND DEDUCTIONS

May R. Berenbaum[1], Michael B. Cohen[1] and Mary A. Schuler[2]

[1]Department of Entomology and [2]Department of Plant Biology
University of Illinois, 505 S. Goodwin
Urbana, IL 61801-3795

INTRODUCTION

Although it is now known that cytochrome P450 monooxygenases have a number of physiological functions in insects, including hormone and pheromone metabolism, they were first recognized as a general purpose system for metabolizing xenobiotics, particularly synthetic organic insecticides. In the early sixties, a series of experiments revealed that high levels of oxidase activity were associated with resistance to insecticides and that administration of known oxidase inhibitors, such as sesamex or piperonyl butoxide, could reverse that resistance. Subsequently, detoxification via microsomal monooxgenases of such insecticides as propoxur, carbaryl, and aldrin was demonstrated. The fact that insect strains selected for resistance to one class of insecticide often displayed resistance to other groups of insecticides suggested that the microsomal monooxygenase system was broadly substrate-specific (Hodgson, 1985). The microsomal cytochrome P450- dependent mono-oxygenase system was subsequently found to consist of a suite of membrane-bound enzymes that effect a variety of oxidation reactions; the heme protein cytochrome P450 is the terminal oxidase in the series. In general, since oxidation increases the hydrophilicity of a substrate, P450 monooxygenases can act as detoxification enzymes; with reduced lipophilicity, a substrate is at the same time rendered more likely to be excreted and, in the process, less able to cross lipid rich membranes to interfere with biological processes.

The fact that virtually all aerobic life on this planet, including many species that never encounter synthetic organic insecticides, possess active P450 monooxygenase systems prompted speculation as to the natural substrates of the xenobiotic-metabolizing P450s. Krieger et al. (1971) proposed the idea that insecticide metabolism via microsomal monooxygenases in foliage-feeding insects was a result of preadaptation; oxidative metabolism of xenobiotics diversified originally to facilitate feeding on the chemically defended tissues of angiosperm host plants. In support of this concept, Krieger et al. (1971) reported that monooxygenase activity, as measured by aldrin epoxidation, is higher in polyphagous species with a broad diet than in monophagous species feeding only on a single plant family. Ostensibly, caterpillars feeding on a variety of plant families are exposed to a wider variety of plant secondary substances and accordingly require higher levels of oxidase activity to utilize such a broad range of hosts. Subsequent work by Brattsten and colleagues (Brattsten 1979a,b; Brattsten et al., 1977) demonstrated that a number of plant secondary substances do indeed induce microsomal monooxygenase activity in caterpillars and that this induction can increase tolerance to other toxic chemicals.

Furanocoumarins are such naturally occurring plant secondary substances. Restricted in distribution to fewer than a dozen plant families, these compounds are most widespread and structurally diverse in the Umbelliferae (Apiaceae), the carrot family (Murray et al., 1982) and the Rutaceae, the citrus family. Furanocoumarins are unusual among natural products in that they are photoactive; absorption of ultraviolet energy enhances their chem-

Molecular Insect Science, Edited by
H. H. Hagedorn *et al.*, Plenum Press, New York, 1990

ical reactivity. Photoactivated furanocoumarins can bind to pyrimidine bases in DNA and cross link opposing strands, effectively preventing transcription and replication (Scott et al., 1976). In addition, they can bind to and denature proteins and form cycloadducts with unsaturated fatty acids. Thus, furanocoumarins are broadly biocidal; toxic effects of furanocoumarin exposure have been reported in yeast, bacteria, virus, molluscs, insects, birds, and mammals (Murray et al., 1982).

ROLE OF P450s IN ADAPTATION TO FURANOCOUMARIN-CONTAINING PLANTS

Despite the general toxicity of furanocoumarins, plants that produce them are susceptible to damage by herbivorous insects. Chief among the insect enemies of furanocoumarin-containing plants in the Umbelliferae are species in the genus *Papilio* (Lepidoptera: Papilionidae). *Papilio polyxenes*, the eastern black swallowtail, feeds on at least 10 genera of umbellifers throughout its range (Tietz, 1972). *P. polyxenes* is extra-ordinarily resistant to the effects of ingesting furanocoumarins. While xanthotoxin (8 methoxypsoralen), a linear furanocoumarin, is antifeedant to the polyphagous noctuid *Spodop-tera litura* at concentrations of 300 ppm (Yajima et al., 1977) and toxic to *Spodoptera eridania* at concentrations of 1000 ppm (Berenbaum, 1978), it is completely nontoxic to black swallowtails at concentrations of 10,000 ppm, or 1% of fresh weight of diet. In fact, at these high concentrations, xanthotoxin actually has a salutary effect on swallowtail growth and development (Berenbaum, 1981). Virtually every recorded host of the black swallowtail is known to contain furanocoumarins.

Papilio polyxenes appears to owe its resistance to furanocoumarins to its extraordinary capacity to metabolize xenobiotics. On its normal host plants, microsomal monooxygenase activity of *P. polyxenes* is extremely high when measured with model substrates (Brattsten, 1979; Neal, 1987). Ivie et al. (1983) established that xanthotoxin is metabolized in this species to two nonphototoxic dicarboxylic acid derivatives. The fact that xanthotoxin metabolism is NADPH-dependent and is inhibited by piperonyl butoxide is consistent with cytochrome P450 involvement (Bull et al., 1984, 1986). We have also established that cytochrome P450s involved in xanthotoxin metabolism are inducible by xanthotoxin. Xanthotoxin concentrations can be increased via topical application to host plant such that P450 activity is increased sevenfold over activity on unsupplemented foliage (Cohen et al., 1989). In addition, an approximately fourfold increase in xanthotoxin metabolic activity is not reflected in a significant increase total P450 content, suggesting that only a small subset of P450 enzymes is involved in xanthotoxin metabolism (Cohen et al., 1989).

Cytochrome P450 metabolism of xanthotoxin in phytophagous insects appears to contravert the contention of Krieger et al. (1971) that polyphagous species possess greater detoxification abilities. In the case of xanthotoxin metabolism, specialists such as the black swallowtail and the parsnip webworm (*Depressaria pastinacella*), an oecophorid caterpillar restricted to two umbelliferous hosts containing furanocoumarin (Nitao, 1989), can process xanthotoxin at rates 30 to 300 times faster than can polyphagous noctuids such as *Trichoplusia ni*, the cabbage looper (Lee, 1989) and Spodoptera eridania (Ivie et al., 1983, 1986). The cabbage looper, *T. ni*, is a generalist noctuid with a host range encompassing over 50 species of plants, including several umbellifers that contain furanocoumarin (it is a minor pest of celery in California--Jones and Granett, 1982); notwithstanding, it cannot tolerate high concentrations of xanthotoxin. Levels of 0.3% in artificial diet effect complete mortality of neonate larvae (Zangerl, 1990), and concentrations of 0.15% significantly reduce growth rate, consumption rate, and digestive efficiencies of fifth instar larvae (Lee, 1989).

Yet another unexpected aspect of P450 metabolism of xanthotoxin in the black swallowtail is its apparent specificity. Cytochrome P450s have long been regarded as "broadly substrate specific" (Ahmad, et. al., 1986) however, activity levels, even in ecological studies, are almost invariably measured on synthetic substrates, an approach that will detect only those forms of P450 generalized enough to metabolize a variety of substrates. In the black swallowtail, induction of xanthotoxin-metabolizing activity is relatively specific; widely used synthetic inducers such as indole-3-carbinol and phenobarbital are ineffective as inducers of xanthotoxin-metabolizing cytochrome P450 (Table 1). In contrast, compounds similar in structure to xanthotoxin (such as its 5-methoxy isomer bergapten) induce activity to varying degrees (Table 2).

High rates of activity, inducibility of metabolic activity by the furanocoumarin substrate (Cohen et al., 1989; Nitao, 1989), and the lack of a corresponding increase in total P450

content with induction of metabolic activity by xanthotoxin (Cohen et al., 1989) all are suggestive of the existence of a P450 isozyme (or isozymes) relatively substrate-specific for compounds present in the hostplants of specialists.

Table 1. Metabolism of xanthotoxin in black swallowtail larvae after exposure to putative inducers

INDUCER	XANTHOTOXIN METABOLIZED[a] (nmoles/mg protein/min)
xanthotoxin	5.03 ± 0.28 a
pentamethylbenzene	1.95 ± 0.13 b
indole-3-carbinol	1.66 ± 0.24 bc
phenobarbital	1.57 ± 0.14 bc
beta-naphthoflavone	1.42 ± 0.17 bc
acetone (control)	1.11 ± 0.21 c

[a]Mean ± SE of two experiments each assayed in triplicate. One-way ANOVA showed a significant effect of diet on xanthotoxin metabolized ($F_{5,30}$ = 50.54, P=0.0001). Means followed by the same letter are not significantly different at P=0.05, pairwise comparisons using LSD. Final instar larvae were reared for 2-3 days on parsley treated with a solution of an inducer in acetone; final concentration in the diet was 0.01 nmoles/g fresh weight. P450 activity was measured as described in Cohen et al. (1989).

Table 2. Metabolism of xanthotoxin in black swallowtail larvae after exposure to plant allelochemicals

INDUCER	XANTHOTOXIN METABOLIZED[a] (nmoles/min/mg protein)
xanthotoxin	5.08 ± 0.18 a
bergapten	4.34 ± 0.51 ab
coumarin	3.92 ± 0.28 abc
flavone	3.75 ± 0.90 c
angelicin	2.86 ± 0.22 cd
acetone (control)	1.68 ± 0.33 d

[a]Mean ± SE of three experiments assayed in triplicate. One-way ANOVA showed a significant effect of diet on xanthotoxin metabolized ($F_{5,39}$ = 9.55, P=0.0001). Means followed by the same letters are not significantly different at P=0.05, pairwise comparisons using LSD. Larvae reared as described in Table 1.

MOLECULAR CHARACTERIZATION OF P450-MEDIATED FURANOCOUMARIN METABOLISM

We are currently using techniques of molecular biology to identify the gene or genes coding for xanthotoxin-metabolizing cytochrome P450 monooxygenases in the black swallowtail. Analysis of swallowtail microsomes by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) demonstrated the existence of a 55 kDa polypeptide band induced by xanthotoxin in a dose-dependent manner; this polypeptide is within the molecular size range of cytochrome P450 monooxygenases (45-60 kDa) (Figure 1). In addition, this 55 kDa band selectively cross-reacts on Western blots with a P450 antibody, anti-rat P450 UT-A (Cohen et al., in preparation). Thus, swallowtail cytochrome P450s, like other insect P450s (Feyerheisen et al., 1989) have conserved regions in common with mammalian P450s.

The 55 kDa polypeptide was separated on a one-dimensional gel, transferred to a polyvinylidene difluoride membrane (Matsudaira, 1987) and partially sequenced (Figure 2). The 14 N-terminal amino acid sequence was compared to all proteins in the National Biomedical Research Foundation Protein Identification Resource data bank. The closest match was with rabbit P450 LM2 (=P450 IIB4). The 55 kDa polypeptide shares a 60% identity with this P450 for the region sequenced. The xanthotoxin-inducible P450 of the

black swallowtail shows no striking similarity to the phenobarbital- inducible P450 characterized from house flies by Feyereisen et al. (1989).

After obtaining a longer sequence, we plan to synthesize degenerate oligonucleotide probes corresponding to the N-terminal amino acids of the 55 kDa polypeptide. A cDNA library has been constructed in the pCGN 1703 plasmid vector (Alexander, 1987), from mRNAs isolated from xanthotoxin-induced swallowtail caterpillar midguts. Cloned cDNAs thus identified will be subcloned, characterized by restriction site and DNA sequence analysis, and compared with the N-terminal sequence of the xanthotoxin-induced P450 as well as other available P450 sequences.

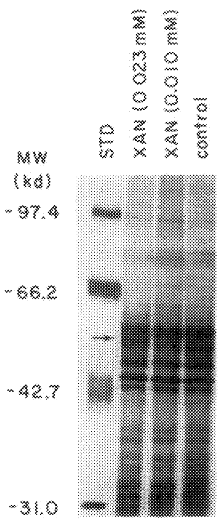


Fig. 1. SDS-PAGE of microsomal proteins from black swallowtail larvae. Final instar larvae were reared for 2-3 days on parsley treated with acetone (control) or xanthotoxin in acetone; final xanthotoxin concentrations in diet were 0.023 or 0.01 mmoles/g fw parsley. Three μg of protein were loaded per lane. The gel was silver stained.

Xanthotoxin-induced P450	MLY-LLALVTFLAGL
	: . :: :... ::::
Rabbit LM2 (=P450IIB4)	MEFSLLLLLAFLAGLLLLL

Figure 2. Amino-terminal sequence comparison. Two dots indicate a shared amino acid, one dot a conservative amino acid substitution.

POTENTIAL APPLICATIONS OF MOLECULAR CHARACTERIZATION OF P450s INVOLVED IN PLANT-INSECT INTERACTIONS

Characterizing the gene(s) regulating P450-metabolism of xanthotoxin in the black swallowtail provides an approach to evaluating a variety of ecological and evolutionary concerns. Plants, and the insects that eat them, constitute approximately half of all life on earth; the evolution of this tremendous diversity has long been a subject for speculation and debate. A current concern in discussions of the evolution of specialization in phytophagous insects revolves around the evolutionary precedence of physiological, as opposed to behavioral, adaptation to novel host plant chemicals. Ehrlich and Raven (1964) speculated that, with sufficient selection pressure resulting from the presence of toxic host plant constituents, a random genetic change conferring resistance to those constituents could allow a major adaptive radiation in the resistant insect taxon. The subfamily Papilioninae, to which the black swallowtail belongs, consists of four tribes; feeding exclusively on

furanocoumarin- containing plants is restricted to the tribe Papilionini, the most speciose of the tribes in the family.

Within the subfamily Papilioninae, P450 activity levels, as measured by O-demethylation of p-nitroanisole (Neal, 1987), vary over two orders of magnitude and are highest in those taxa associated with furanocoumarin-containing plants; *P. cresphontes*, the orange dog swallowtail, which feeds on rutaceous plants, and *P. polyxenes*, which feeds on both rutaceous and umbelliferous herbaceous plants. Hancock (1983) has suggested that Rutaceae-feeding has arisen at least three times in the genus Papilio. It would be instructive to examine members of the tribe for the presence of the gene or genes regulating P450s involved in furanocoumarin metabolism. The extent of homology within the tribe among members whose hosts do produce furanocoumarins can give an indication as to whether furanocoumarin metabolism is a shared derived trait which led, as Ehrlich and Raven (1964) postulated, to an adaptive radiation, or whether it arose several times as the result of parallel evolution of different ancestral P450s.

The cloning of a P450 gene responsible for allelochemical detoxification in a specialist has applied implications as well as theoretical value. The existence of a specialized detoxification pathway gives rise to the possibility of designing a specific inhibitor for that pathway. Such an inhibitor would be beneficial in two ways; it would presumably have low impact on nontarget species and it would render toxic certain chemicals already present in plants, obviating the need for additional environmental inputs of potentially toxic or disruptive substances. Although specific inhibitors have been designed for use against P450s that metabolize endogenous substrates, design of such inhibitors against P450 xenobiotic-metabolizing enzymes has been less successful. However, there is evidence that this approach may be successful; bergapten, a naturally occurring isomer of xanthotoxin, administered *in vitro* to microsomes of parsnip webworm can inhibit xanthotoxin metabolism by more than 60% (A. Zangerl, personal communication). Control efforts incorporating a P450 specific inhibitor are consistent with current objectives in pest management to reduce inputs of hazardous chemicals. By interfering with the ability of a specialist insect to process plant secondary compounds, the naturally occurring toxins already present in plant tissue can be rendered more effective--back to nature, as it were, through biotechnology.

ACKNOWLEDGMENTS

This work was supported in part by an NSF Presidential Young Investigator's Award to MRB and by NSF BSR 88-18205 to M.R. Berenbaum and A. Zangerl and by NSF DCB 84-02792 to M.A. Schuler. We thank F.P. Guengerich for the gift of numerous P450 antisera.

REFERENCES

Ahmad, S., Brattsten, L., Mullin, C. and Yu, S. 1986. Enzymes involved in the metabolism of plant allelochemical. pp. 73-151. in: "Molecular Aspects of Insect-Plant Interactions." Brattsten, L and Ahmad, S., eds. Plenum Press, NY.

Alexander, D.C. 1987. An efficient vector-primer cDNA cloning system. Meth. Enzymol. 154: 41-64.

Berenbaum, M. 1978. Toxicity of a furanocoumarin to armyworms: A case of biosynthetic escape from insect herbivores. Science 201: 532-534.

Berenbaum, M. 1981. Effects of linear furanocoumarins on an adapted specialist insect (*Papilio polyxenes*). Ecol. Entomol. 6: 345-351.

Brattsten, L.B. 1979a. Biochemical defense mechanisms in herbivores against plant allelochemicals. pp. 199-270 in: "Herbivores: Their Interactions with Plant Secondary Metabolites." Rosenthal, G.A. and Janzen, D.H., eds. Academic Press, New York.

Brattsten, L.B. 1979b. Ecological significance of mixed-function oxidations. Drug Metab. Rev. 10: 35-58.

Brattsten, L.B., Wilkinson, C.F. and Eisner, T. 1977. Herbivore- plant interactions: Mixed-function oxidases and secondary plant substances. Science 196: 1349-1352.

Bull, D.L., Ivie, G.W., Beier, R.C., Pryor, N.W. and Oertli, E.H. 1984. Fate of photosensitizing furanocoumarins in tolerant and sensitive insects. J. Chem. Ecol. 10: 893-911.

Bull, D.L., Ivie, G.W., Beier, R.C. and Pryor, N.W. 1986. *In vitro* metabolism of a linear furanocoumarin (8-methoxypsoralen, xanthotoxin) by mixed-function oxidases of larvae of black swallowtail butterfly and fall armyworm. J. Chem. Ecol. 12: 885- 892.

Cohen, M.B., Berenbaum, M.R. and Schuler, M.A. 1989. Induction of cytochrome P450-mediated metabolism of xanthotoxin in the black swallowtail. J. Chem. Ecol. 15: 2347-2355.

Ehrlich, P. and Raven, P. 1964. Butterflies and plants: a study in coevolution. Evol. 18: 586-608.

Feyereisen, R., Koener, J.F., Farnsworth, D.E. and Nebert, D.W. 1989. Isolation and sequence of a cDNA encoding a cytochrome P- 450 from an insecticide-resistant strain of the house fly, *Musca domestica*. Proc. Natl. Acad. Sci. USA 86: 1465-1469.

Hancock, D.L., 1983. Classification of the Papilionidae: a phylogenetic approach. Smithersia 2: 1-48.

Hodgson, E. 1985. Microsomal mono-oxygenases. pp. 225-321 in: "Comprehensive Insect Physiology, Biochemistry, and Pharmacology." vol. II. Kerkut, G.A. and Gilbert, L.I. eds. Pergamon Press, NY.

Ivie, G.W., Bull, D.L., Beier, R.C., Pryor, N.W. and Oertli, E.H. 1983. Metabolic detoxification: Mechanism of insect resistance to plant psoralens. Science 221: 374-376.

Jones, D. and Granett, J. 1982. Feeding site preferences of seven lepidopterous pests of celery. J. Econ. Ent. 75: 449-453.

Krieger, R.I., Feeny, P.O. and Wilkinson, C.F. 1971. Detoxication enzymes in the guts of caterpillars: An evolutionary answer to plant defenses. Science 172: 579-581.

Lee, K. 1989. Enzymatic defenses against plant phototoxins in phytophagous insects. Ph.D. Dissertation. University of Illinois, Urbana-Champaign.

Matsudaira, P. 1987. Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. J. Biol. Chem. 262: 10035-10038.

Murray, R.D.M., Mendez, J. and Brown, S.A. 1982. "The Natural Coumarins." J. Wiley and Sons Ltd., Chichester.

Neal, J.J. 1987. Ecological aspects of insect detoxification enzymes and their interaction with plant allelochemicals. Ph.D. Dissertation. University of Illinois, Urbana-Champaign.

Nitao, J.K. 1989. Enzymatic adaptation in a specialist herbivore for feeding on furanocoumarin-containing plants. Ecol. 70: 629- 635.

Scott, B.R., Pathak, M.A. and Mohn, G.R. 1976. Molecular and genetic basis of furocoumarin reactions. Mutation Research 39: 29-74.

Tietz, H.M. 1972. "An Index to the Described Life Histories, Early Stages, and Hosts of the Macrolepidoptera of the Continential United States and Canada." Allyn Museum of Entomology, Sarasota, Florida.

Yajima, T., Kato, N. and Munakata, K. 1977. Isolation of insect antifeeding principles in *Orixa japonica*. Agric. Biol. Chem. 41: 1263-1268.

Zangerl, A. 1990. Furanocoumarin induction in wild parsnip: Evidence for an adaptive induced defense. Ecology (in press).

BIOCHEMISTRY AND MOLECULAR BIOLOGY OF INSECT CYTOCHROME P450

R. Feyereisen, J. F. Koener, F. A. Cariño
and A. S. Daggett

Department of Entomology
Oregon State University
Corvallis, Oregon 97331

INTRODUCTION

Cytochrome P450 monooxygenases (P450) are a diverse and ubiquitous group of enzymes encoded by genes from a superfamily comprising at least 17 families, and probably many more (Nebert and Gonzalez, 1987; Nebert et al., 1989). Our present understanding of P450 enzymes comes mainly from studies of P450 in vertebrates, where they are involved in steroid biosynthesis and in the metabolism of drugs and chemical carcinogens, and in bacteria, with P450*cam* from *Pseudomonas putida* being the only P450 enzyme for which a crystal structure is presently available (Poulos et al., 1987).

Work of Fenwick (1958), Agosin et al. (1961) and Arias and Terriere (1962) on the enzymology of insect microsomal oxidases, followed by the demonstration of insect microsomal P450 by Ray (1965), constitute the early, historical landmarks of insect P450 research. The next quarter of a century of research, exhaustively reviewed by Agosin (1985) and Hodgson (1985), focused on the role of insect P450 in the detoxification of insecticides with an emphasis on insecticide resistance and on insecticide synergists, particularly methylenedioxyphenyl (MDP) compounds.

New aspects of P450 research in insects have emerged during the last ten years. These include the discovery that P450 enzymes are involved in insect hormone and pheromone metabolism, and the realization that the microsomal P450 enzymes, so well suited to foil the chemist's attempt to kill insects, have evolved over the last several hundred million years as an adaptation of insects to the wide variety of potentially toxic components of their diet and are often inducible by plant chemicals.

Diversity of P450 structure and function

The number of different P450 proteins present in any one insect species is not known. Ronis et al. (1988) were able to separate at least 7 forms of P450 from adult house flies, and there are at least 32 distinct P450 genes in the rat. It would not be surprising, therefore, to find several dozen P450 genes in any one insect species. This diversity of P450 genes offers a great latitude for the rapid adaptation to new chemical stresses such as an insecticide in the short term or chemicals found in a new host plant in the long term. When such genetic diversity is coupled with inducibility of P450 gene expression by foreign or endogenous chemicals and is controlled by developmental cues, the qualitative and quantitative changes in P450 activities are bound to have important toxicological consequences.

We will review here some of the common features of insect and vertebrate P450 systems, then briefly highlight some specific aspects of insect P450 which we believe to be of interest. We will not draw particular attention to the huge and obvious gaps in our knowledge of insect P450.

Molecular Insect Science, Edited by
H. H. Hagedorn *et al.*, Plenum Press, New York, 1990

BIOCHEMISTRY OF P450 ENZYME SYSTEMS

The theoretical stoichiometry of cytochrome P450 monooxygenase reactions is:

$$AH + O_2 + 2e^- + 2H^+ \dashrightarrow AOH + H_2O$$

Insertion of one atom from molecular dioxygen into the substrate AH and reduction of the second atom to water is the mixed-function referred to in the common name "mixed-function oxidase" (MFO) coined originally by Mason, and not to the fact that substrate specificity can be broad for some P450 proteins (a characteristic captioned by the term "polysubstrate monooxygenase" or PSMO). The source of reducing equivalents is NADPH, and the microsomal enzyme NADPH:cytochrome P450 reductase (EC 1.6.2.4), a flavoprotein with one FAD and one FMN prosthetic group, is responsible for the transfer of electrons, one at a time, to P450 proteins. Antibodies to purified insect P450 reductase have clearly demonstrated the role of this flavoprotein in P450-catalyzed reactions, including microsomal hydroxylation of ecdysone to 20-hydroxyecdysone (Crankshaw et al., 1981; Feyereisen and Vincent, 1984; Halliday et al., 1986). The role of cytochrome b_5 and NADH:cytochrome b_5 reductase in providing one of the two electrons for some P450 reactions has not yet been tested convincingly in insects. Similarly, the electron transfer chain to mitochondrial P450 enzymes (e.g. in ecdysteroid metabolism, the 22-hydroxylase in prothoracic glands or the 2- and the 20-hydroxylases in certain peripheral tissues) has not been characterized, and thus may or may not be homologous to the adrenodoxin/adrenodoxin reductase system found in mammalian mitochondrial P450 systems.

Table 1: Molar ratio of P450 and NADPH:cyt P450 reductase in microsomal membranes from insects:

SPECIES / TISSUES	MOLAR RATIO P450/REDUCTASE
Musca domestica larvae	17
Musca domestica adult abdomens	18
Culex pipiens adult fat body	14
Diploptera punctata larval midgut	11
Locusta migratoria larvae	
Malpighian tubules	8
fat body	15
Spodoptera eridania larvae	
midgut (control)	6
midgut (diet + limonene)	11

P450 proteins are present in the endoplasmic reticulum membrane in molar excess to the P450 reductase (Table 1). This "excess" of P450 protein would require an efficient coupling of P450 and P450 reductase in the endoplasmic reticulum membrane. The estimated collision rate of P450 proteins and P450 reductase is 10^5-10^6 times higher than the turnover number of P450-catalyzed reactions (usually << 1/sec). This would argue that the transfer of electrons to P450 is not "rate-limiting." However, thromboxane synthetase, a P450 enzyme which does not require O_2 or reducing equivalents from NADPH, or $P450_{BM-3}$ (P450 CII) which is a fusion protein between P450 reductase and P450 (Ruettinger et al., 1989) have turnover numbers that are 10^3 times higher than microsomal or mitochondrial P450 systems. In addition, antibodies to P450 reductase block monooxygenase reactions more readily than they block reduction of cytochrome c (Feyereisen and Vincent, 1984).

The supply of, and competition for, reducing equivalents from NADPH thus remains an important consideration in any discussion of microsomal P450 activity.

Reactions catalyzed by insect P450 enzymes

The variety of reactions catalyzed by P450 enzymes is astonishing, and the major types of reactions are summarized here according to the classification of Ortiz de Montellano (1986).

Hydrocarbon hydroxylation: Examples include the hydroxylations of DDT to dicofol and ecdysone to 20-hydroxyecdysone. O-dealkylation reactions which result from an initial hydroxylation are also well documented. Examples include alkoxyresorufin or alkoxycoumarin O-dealkylations and p-nitroanisole O-demethylation, which are useful fluorometric or spectrophotometric assays for P450 activity.

Olefin epoxidation: The epoxidation of the cyclodiene insecticide aldrin to dieldrin has probably been used most extensively in studies of insect P450. An example of epoxidation of an endogenous substrate is the conversion of methyl farnesoate to juvenile hormone III in insect corpora allata.

Aromatic hydroxylations: There may be two types of aromatic hydroxylations, either an initial epoxidation followed by an opening of the epoxide ring and an "NIH shift," or less frequently a direct hydroxylation. Examples include the hydroxylation of the carbamate insecticides carbaryl and propoxur, as well as naphthalene and biphenyl hydroxylations.

Heteroatom (S- and N-) oxidation or dealkylation: These reactions result in the formation of N-oxides (of plant alkaloids) and S-oxides (sulfoxides and sulfones of several pesticides). N-dealkylations are well documented in insects (N-methyl-p-chloroaniline is often used as model substrate) but S-dealkylation has only recently been documented using the substrate 6-methylthiopurine (Yu, 1988). The initial oxygenation of phosphorothioate insecticides and their subsequent conversion to phosphates with concomitant desulfuration or phosphate ester cleavage can also be classified in this category. Initial oxygenation to an S-oxide is viewed as leading to an intermediate phosphooxythirane which subsequently decomposes. The P=S to P=O conversion is a major bioactivation step, which greatly increases the anticholinesterase activity of these zoocides. Several N- and S-oxidation reactions can be catalyzed by a microsomal FAD-monooxygenase (O_2 and NADPH-dependent) in mammalian species, but this enzyme has not been reported from insects.

Other types of reactions known to be catalyzed by P450 enzymes in other systems have not been described to date in insects. They include C-C bond cleavage as in the side-chain cleavage of cholesterol, the progesterone 17, 20-lyase activity, the aromatization of androgens, or the 14-demethylation of lanosterol. These reactions are usually the culmination of sequential oxygenation reactions. Conversion of C29 or C28 phytosterols to C27 sterols is known to occur in insects, but a role for P450 enzymes in these reactions has not been demonstrated.

A single P450 protein can catalyze several types of reactions, e.g. epoxidation and hydroxylation, and one P450 protein can also oxygenate a single substrate at several positions. Whereas the ferrocytochrome-CO complex allows the spectrophotometric measurement of the sum of all P450 proteins in microsomes, there is presently no way to estimate the activity of individual types of P450 proteins in their membrane environment. Use of a single "model" substrate can be misleading, and the development of biochemical assays for the metabolism of target chemicals would be preferable in studies of insecticide resistance or plant allelochemical effects.

MOLECULAR BIOLOGY OF INSECT P450

The cloning and sequencing of a house fly P450 cDNA (Feyereisen et al., 1989) now allow a direct comparison of an insect P450 enzyme with other P450 enzymes which are better understood. The alignment of the house fly P450 (named P450 VIA1 in the new P450 nomenclature, gene name *CYP6A1*; Nebert et al., 1989) with all known P450 proteins showed the greatest similarity (27%) with members of family III. This level of similarity indicates that house fly P450 VIA1 has diverged from other microsomal P450s more than family I (which includes the aryl hydrocarbon-inducible P450 proteins) has diverged from family II (which includes many phenobarbital-inducible P450 proteins). Consequently, a

comparison of insect P450 with vertebrate "phenobarbital-inducible" or "aryl hydrocarbon-inducible" P450 based on spectral data or substrate preference has little meaning. Hydropathy plots show that all microsomal P450 proteins (MW: 50-60,000) have a hydrophobic N-terminus which encompasses one or possibly two transmembrane segments, with the bulk of the protein being on the cytoplasmic side of the membrane. Other important domains of P450 proteins include the heme binding site and the substrate binding site.

Conserved heme binding domain

This region located towards the C-terminus of all P450 proteins contains the Cys residue providing the fifth (axial) ligand of the heme iron. Sequences of all P450 proteins described to date show an invariant F**G***C*G sequence which is not found in other proteins, and which thus constitutes the P450 "signature." Fourteen residues of this conserved Cys peptide are aligned in Table 2 and show that P450 VIA1 has 12/14 residues in common with P450 IIIA2, although it is only 27% similar over the full length of the protein. A genomic P450 clone from *Aedes aegypti* was also identified by the presence of such a typical Cys peptide (Bonet et al., 1989). Direct evidence for the role of this conserved Cys residue comes from the analysis of the crystal structure of bacterial P450*cam* (P450 CI) which showed that Cys-357 is the axial thiolate ligand (Poulos et al., 1987). The conserved Gly-353 initiates a hairpin turn allowing a short antiparallel beta-pair to bracket the heme ("Cys ligand loop"). Shimizu et al. (1988) confirmed these structural studies and showed by site-directed mutagenesis of P450 IA2 that CO binding was lost in Cys-456 to Tyr/His and in Gly-458 to Glu mutants.

Table 2: Alignment of the conserved heme binding domain in P450 proteins:

P450 family	*			*				*		*				
VIA1	F	G	D	G	P	R	N	C	I	G	M	R	F	G
								449						
CI (*cam*)	F	G	H	G	S	H	L	C	L	G	Q	S	L	A
								357						
IA2 (d)	F	G	L	G	K	R	R	C	I	G	E	I	I	G
								456						
IIB4	F	S	L	G	K	R	I	C	L	G	E	G	I	A
IIIA2	F	G	N	G	P	R	N	C	I	G	M	R	F	A
IVA1	F	S	G	G	A	R	N	C	I	G	K	Q	F	A
XIA	F	G	W	G	V	R	Q	C	V	G	R	R	I	A
XVIIA1	F	G	A	G	P	R	S	C	V	G	E	M	L	A
XIXA1	F	G	F	G	P	R	G	C	A	G	K	Y	I	A
XXIA1	F	G	C	G	A	R	V	C	L	G	E	P	L	A
XXVIA1	F	G	Y	G	V	R	A	C	L	G	R	R	I	A
LI	F	G	G	G	R	H	R	C	I	G	E	H	F	A
LIIA1	F	N	G	G	P	R	I	C	L	G	Q	Q	F	A
CII	F	G	N	G	P	R	N	C	I	G	M	R	F	A
CIII	F	G	A	G	V	H	R	C	L	G	E	T	L	A
CIV	F	G	S	G	P	H	H	C	P	G	A	Q	I	S

Oxygen / substrate binding site

This domain of the P450 molecule constitutes the region distal (opposite) the heme. It is characterized by a highly conserved Thr residue (Thr-309 in P450 VIA1) (Table 3). The crystal structure of P450*cam* (Poulos et al., 1987) indicates a local distortion of helix I of P450*cam* at Thr-252 where oxygen binds when it coordinates the heme iron. Moreover, the conserved Thr is preceded by several hydrophobic residues in most P450 families including family VI and these residues contact the substrate camphor in P450*cam*. Site-directed mutagenesis of several residues around the conserved Thr-319 of P450 IA2 resulted in a

marked change in the binding affinity of axial ligands coordinating the heme iron (e.g. imidazoles, metyrapone, aniline and pyridine) (Shimizu et al., 1989). Significant changes in the k_{cat}/K_m toward various substrates were obtained in mutants of this conserved Thr or neighboring residues (Furuya et al., 1989; Imai and Nakamura, 1989), thus confirming that this region is a part of the substrate binding site. Other parts of the P450 molecule are also involved in substrate binding (Lindberg et al., 1989), and a clear understanding of the relationship between structure and substrate specificity is still distant. The catalytic activity of house fly P450 VIA1 has not yet been characterized by expression of the cDNA in a suitable system such as transformed yeast or higher eukaryotic cell lines.

Table 3: Alignment of the substrate binding region of P450 proteins:

	* (above T)
VIA1	F V F F L G G F E T S S S T M G F
	309
CI (*cam*)	G L L L V G G L D T V V N F L S F
	252
IA2 (d)	N D I F G A G F E T V T T A I F W
	319
IIB4 (LM2)	L S L F F A G T E T T T S T T L R
	302
IIIA2	V I F I F A G Y E T T S S T L S F
	310
XIA (scc)	T E M L A G G V N T T S M T L Q W
	329
XXI (C21)	V D L F I G G T E T T A S T L S W
	294
LIIA1	L N I L V A G R D T T A G L L S F
	340

Hydrophobic residues preceding the conserved Thr residue are underlined

P450 interaction with P450 reductase

The interaction of P450 proteins with NADPH:cytochrome P450 reductase (EC 1.6.2.4) is believed to result from electrostatic contacts between the two proteins, with P450 reductase conserved carboxylates (Glu, Asp) interacting with conserved basic residues (Lys, Arg) of P450 proteins. This view emerged from the study of P450 reductase interaction with cytochrome c which can serve as an artificial electron acceptor (Nisimoto, 1986), and a putative P450 reductase recognition site was identified in Nelson and Strobel's (1988) study of 34 aligned P450 sequences. Electrostatic contacts are also believed to be used in mitochondrial P450 systems, i.e. the interaction of P450*scc* with adrenodoxin (Tsubaki et al., 1989), as well as the interaction of P450*cam* with putidaredoxin or cytochrome b_5 (Stayton et al., 1989).

A house fly P450 reductase cDNA has recently been sequenced (Koener et al., unpublilshed results) and Table 4 shows the alignment of a portion of the FMN binding domain of the fly enzyme with that of rat, yeast (*Saccharomyces cerevisiae*) and *Bacillus megaterium* $P450_{BM\text{-}3}$. Overall, the amino acid sequence similarity of about 60% between fly and mammalian P450 reductase reflects the constraints on the evolution of the protein imposed by the maintenance of several structural domains: a membrane spanning domain, a cytochrome P450 binding domain, an FMN binding domain, an FAD binding domain, and an NADPH binding domain, as well as the maintenance of the structure needed to allow a correct interaction of these domains.

In the rat, the two flanking Tyr residues of this domain (Tyr-140 and Tyr-178) have been shown by site-directed mutagenesis studies to participate in FMN binding (Shen et al., 1989), and the aromatic side chains are believed to sandwich the isoalloxazine ring of FMN. The cluster of acidic residues (E,D) in the first segment of the alignment of the reductase is

believed to provide the electrostatic interactions with the region surrounding Arg-367 of P450 VIA1. Antibodies to fly NADPH: cytochrome P450 reductase (Feyereisen and Vincent, 1984) inhibit the reduction of ferricyanide very poorly, but are potent inhibitors of all P450 reactions. Since the reduction of ferricyanide by the reductase does not require FMN, but involves a direct reduction from FAD, it seems likely that this region of the reductase is more strongly antigenic than the NADPH or FAD binding domains.

Table 4: Alignment of the FMN binding region of NADPH:cytochrome P450 reductase (identical residues indicated by a dot).

```
          house fly   YGEGDPTDNAMEFYEWITNGEVD-LTGLNYAVFGLGNKTY

          rat                   QD  D LQETD      VKF

          yeast            FP G VN EDF C A AGA SN R NM     S

bacterial P450BM-3    .-N.H.P...KQ.VD.LDQASA.EVK.VR.S...C.D.NW
```

ENDOGENOUS SUBSTRATES VS XENOBIOTICS

A clear distinction between the P450 proteins responsible for the metabolism of endogenous substrates and those metabolizing foreign chemicals is difficult to justify because a single amino acid modification can convert a coumarin hydroxylase into a testosterone hydroxylase and vice versa (Lindberg et al., 1989). Rapid evolution of a new "xenobiotic"-metabolizing P450 from e.g. a steroid-metabolizing P450 following gene duplication is therefore plausible. Moreover, a distinction between "mitochondrial" and "microsomal" P450 systems has merit only with respect to the structure of the electron-tranfer chain to P450, but not with respect to the substrates of P450 as has sometimes been suggested. The presence of inducible mitochondrial P450 capable of metabolizing exogenous substrates such as aflatoxin B_1 has been demonstrated (Shayiq and Avadhani, 1989) and mitochondrial P450 proteins thus do not metabolize endogenous substrates exclusively. The role of microsomal P450 proteins in metabolizing endogenous substrates has been clear for some time, even in insects (ecdysone, methyl farnesoate, Z-9-tricosene). Thus, the difference between mitochondrial and microsomal P450 may be less fundamental than had been thought.

The discovery of a considerable degree of homology between sulfite reductase from *E. coli* and *Salmonella typhimurium* (Ostrowski et al., 1989), and NADPH:cyt P450 reductase (Porter and Kasper, 1985,1986) and the structure elucidation of a fusion protein between P450 and P450 reductase in *Bacillus megaterium* ($P450_{BM-3}$, Ruettinger et al., 1989) leads us to reject the notion that a functional P450 reductase is found only in eukaryotes. The mitochondrial P450 electron transfer chain homologous to the P450*cam* system of *Pseudomonas putida* may simply reflect the closer evolutionary relation of *P. putida* to the alpha-purple bacterial ancestor of mitochondria. It no longer argues for an antecedence of the mitochondrial/P450*cam* system over the microsomal system.

P450 AND INSECT-HOST INTERACTIONS

An artificial distinction between endogenous and xenobiotic substrates may also become moot when one considers that the diet provides most P450 substrates, and that the metabolic "choices," detoxification, utilization as food, precursor for hormone or pheromone synthesis, follow no rules of chemistry or phylogeny. For instance, alpha-pinene is converted to a pheromone in some bark beetles (White et al., 1981) but is "detoxified" in other species. The complex interactions of plant chemicals with insect P450 enzymes (induction, inhibition, metabolism) play a role in the evolutionary ecology of insect-plant interactions (Brattsten, 1979; Brattsten and Ahmad, 1986; Berenbaum, this volume).

Particularly appealing in this regard was the observation by Krieger et al. (1971) of a higher midgut microsomal P450 enzyme activity (aldrin epoxidation) in polyphagous lepidoptera than in monophagous species. A more extensive study of Rose (1985) shows that such a generalization is incorrect, most probably because a single "indicator reaction" such as the epoxidation of aldrin to dieldrin does not, and cannot, give an accurate picture of the diversity of P450 enzymes. Nonetheless, the study of insect-plant interactions probably will provide amazing examples of the adaptive evolution of P450 enzymes (Berenbaum, this volume). Whether P450 enzymes are also involved in the evolutionarily more recent interactions between insects and vertebrate hosts is unclear at this time, mainly because of a paucity of data (Baldridge and Feyereisen, 1986).

P450 AND INSECTICIDE RESISTANCE

The study of the molecular genetics of P450 in insecticide resistance has just begun. Classical biochemical studies have indicated that resistant strains are often characterized by elevated P450 levels and elevated P450 enzyme activities. Moreover, the spectral characteristics of P450 in resistant strains are often different from those in susceptible strains (Agosin, 1985; Hodgson, 1985). An increase in the level of some P450 protein(s) best explains those biochemical data. Indirect data using antibodies to $P450_{Lpr}$ from the house fly suggest that $P450_{Lpr}$ increases from about 7% (in a susceptible strain) to about 70% of the total microsomal P450 in the pyrethroid-resistant Lpr strain (Wheelock and Scott, 1989). In some cases of insecticide resistance, it is a *lower* activity of one or more P450 proteins which contributes to resistance, e.g. when conversion of a phosporothioate to a phosphate (activation step) is reduced (Konno et al., 1989).

Table 5: A molecular classification of insecticide resistance mechanisms

MOLECULAR MECHANISM OF INSECTICIDE RESISTANCE	EXAMPLE
DNA amplification	esterase 4 in *Myzus persicae* esterase B1 in *Culex quinquefasciatus*
Point mutation in structural gene	acetylcholinesterase insensitivity in insects and mites
Change in regulatory gene	chromosome II R gene in *Musca* (chr. III R gene in *Drosophila*) control of P450

To date there is no molecular explanation for the teleologically pleasing idea that the expression of a P450 best suited for detoxification is selectively increased in resistant strains. Genetic studies in both *Musca domestica* and *Drosophila melanogaster* have led to the theory that a regulatory locus is responsible for the increased expression of some P450 genes and genes for other detoxification enzymes such as glutathione S-transferases (Plapp, 1984, Waters and Nix, 1988). Preliminary data in some resistant strains of *Musca domestica* (Cariño et al., unpublished results) and *Anopheles stephensi* (Ladonni et al., 1989) indicate a higher level of specific P450 mRNA. In *M. domestica*, the elevated *CYP6A1* mRNA level is not the result of gene amplification. If the regulatory gene hypothesis of Plapp is proven correct, there would be at least three distinct molecular mechanisms of insecticide resistance (Table 5). These mechanisms do not match with the common physiological/biochemical categories of resistance (behavioral resistance; reduced penetration; increased detoxification; modification of the target site). The development of strategies for the management of insecticide resistance requires a full understanding of both the biochemical and molecular mechanisms of resistance. In the case of esterase gene amplification for instance, a recent study dramatically demonstrated that only a DNA diag-

nostic test could distinguish between aphid revertants (which can easily be re-selected for high resistance) and true susceptible individuals (Field et al., 1989).

P450 INHIBITORS AND THE DESIGN OF "BIORATIONAL" INSECTICIDES

The insecticide synergist piperonyl butoxide, a synthetic ingredient of many "natural" pyrethrin sprays, is perhaps the best known inhibitor of insect P450 reactions. This methylenedioxyphenyl compound is widely used as a diagnostic tool for the involvement of P450 enzymes in insecticide metabolism and/or resistance. Piperonyl butoxide does not inhibit all P450 enzymes equally, however, and the use of a single "generic" inhibitor to quantify the contribution of P450 in resistance suffers from the same limitations as the use of a single "generic" substrate such as aldrin.

When molecules with structural functionalities known to inhibit P450 reactions (methylenedioxyphenyl, imidazole, propynyloxy-, cyclopropylamine, etc.) are screened against P450 enzymes responsible for hormone or pheromone metabolism, specific inhibitors may be found. Such inhibitors may be viewed as potential insect control agents capable of disrupting insect communication, development or reproduction. There are precedents for an approach targeting P450 enzymes. P450 inhibitors with therapeutic or agricultural use include the fungicide miconazole, which inhibits lanosterol 14-demethylase (P450 LII); metyrapone, an inhibitor of the steroid 11-beta hydroxylase (P450 XIB); ancymidol, an inhibitor of ent-kaurene oxidation in the gibberellin biosynthetic pathway; and dazoxiben which has antithrombic activity and inhibits thromboxane synthetase. Although these inhibitors are not rigorously specific for their targeted P450 and have been shown to inhibit other P450 reactions in vitro, their use can be justified by considerations of both physiological and ecological selectivity. P450 inhibitors targeted at juvenile hormone biosynthesis (Feyereisen, 1987) or ecdysone biosynthesis (Burger et al., 1987) have already been evaluated, mainly in vitro, and their study can only increase as new, insect specific, biosynthetic P450 enzymes are characterized.

ACKNOWLEDGMENTS

Supported by NIH grant GM 39014. Oregon Agricultural Experiment Station technical paper no 9078.

REFERENCES

Agosin, M. 1985. Role of microsomal oxidations in insecticide degradation. pp 647-712 in: "Comprehensive Insect Physiology, Biochemistry and Pharmacology." Vol.12. Kerkut, G. A. and Gilbert, L. I., eds. Pergamon Press, Oxford.

Agosin, M., Michaeli, D., Miskus, R., Nagasawa, S. and Hoskins, W. 1961. A new DDT-metabolizing enzyme in the german cockroach. J. Econ. Ent. 54: 340-342.

Arias, R. O. and Terriere, L. C. 1962. The hydroxylation of naphthalene-C14 by housefly microsomes. J. Econ. Ent. 55: 925-929.

Baldridge, G. D. and Feyereisen, R. 1986. Blood meal and cytochrome P-450. Pest. Biochem. Physiol. 25: 407-413.

Bonet, R. G., Crampton, J. and Townson, H. 1989. Mosquito P-450 genes and pyrethroid insecticide resistance. pp in "Molecular Insect Science." Hagedorn, H. H., Hildebrand, J. G., Kidwell, M.G. and Law, J. H., eds. Plenum, New York..

Brattsten, L. B. 1979. Ecological significance of mixed-function oxidations. Drug Metab. Rev. 10: 35-58.

Brattsten, L. B. and Ahmad, S. 1986. "Molecular aspects of insect-plant interactions." Plenum Press, New York.

Burger, A. 1987. *In vitro* studies on potential selective and irreversible inhibitors of enzymes involved in the biosynthesis of ecdysone. Pest. Biochem. Physiol. 29: 197-208.

Fenwick, M. L. 1958. The production of an esterase inhibitor from schradan in the fat body of the desert locust. Biochem. J. 70: 373-381.

Feyereisen, R. 1987. Chemical disruption of insect juvenile hormone biosynthesis. pp 113-116 in: "Pesticide Science and Biotechnology." Greenhalgh, R and Roberts, T. R. eds. Blackwell Scientific Publications, Oxford.

Feyereisen, R. and Vincent, D. R. 1984. Characterization of antibodies to house fly NADPH-cytochrome P-450 reductase. Insect Biochem. 14: 163-168.

Feyereisen, R., Koener, J. F., Farnsworth, D. E., and Nebert, D. W. 1989. Isolation and sequence of cDNA encoding a cytochrome P-450 from an insecticide-resistant strain of the house fly, *Musca domestica*. Proc. Natl. Acad. Sci. USA 86: 1465-1469.

Field, L. M., Devonshire, A. L., ffrench-Constant, R.H., and Forde, B. G. 1989. The combined use of immunoassay and a DNA diagnostic technique to identify insecticid-resistant genotypes in the peach-potato aphid, *Myzus persicae*. Pest. Biochem. Physiol. 34: 174-178.

Furuya, H., Shimizu, T., Hirano, K., Hatano, M., Fuji-Kuriyama, Y., Raag, R., and Poulos, T. L. 1989. Site-directed mutageneses of rat liver cytochrome P-450d: Catalytic activities toward benzphetamine and 7-ethoxycoumarin. Biochem. 28: 6848-6857.

Halliday, W. R., Farnsworth, D. E. and Feyereisen, R. 1986. Hemolymph ecdysteroid titer and midgut 20-monooxygenase activity during the last larval stage of *Diploptera punctata*. Insect Biochem. 16: 627-634.

Hodgson, E. 1985. Microsomal mono-oxygenases. pp 225-321 in: "Comprehensive Insect Physiology, Biochemistry and Pharmacology." Vol 11. Kerkut, G. A. and Gilbert, L. I., eds. Pergamon Press, Oxford.

Imai, Y. and Nakamura, M. 1989. Point mutations at threonine-301 modify substrate specificity of rabbit liver microsomal cytochromes P-450. Biochem. Biophys. Res. Comm. 158: 717-722.

Konno, T., Hodgson, E. and Dauterman, W. C. 1989. Studies on methyl parathion resistance in *Heliothis virescens*. Pest. Biochem. Physiol. 33: 189-199.

Krieger, R. I., Feeny, P. P., and Wilkinson, C. F. 1971. Detoxification enzymes in the guts of caterpillars: An evolutionary answer to plant defenses? Science 172: 579-581.

Ladonni, H. and Townson, H. 1989. Genetics of P-450 mediated pyrethroid resistance in the mosquito *Anopheles stephensi*. pp in: "Molecular Insect Science." Hagedorn, H. H., Hildebrand J. G., Kidwell, M. G., and Law, J. H., eds. Plenum, New York.

Lindberg, R. L. P. and Negishi, M. 1989. Alterations of mouse cytochrome P450coh substrate specificity by mutation of single amino-acid residue. Nature 339: 632-634.

Nebert, D. W. and Gonzalez, F. J. 1987. Structure, evolution and regulation. Ann. Rev. Biochem. 56: 945-993.

Nebert, D. W., Nelson, D. R., and Feyereisen, R. 1989. Evolution of the cytochrome P450 genes. Xenobiotica 19: 1149-1160.

Nelson, D. R. and Strobel, H. W. 1988. On the membrane topology of vertebrate cytochrome P-450 proteins. J. Biol. Chem. 263: 6038-6050.

Nisimoto, Y. 1986. Localization of cytochrome c-binding domain on NADPH-cytochrome P-450 reductase. J. Biol. Chem . 261: 14232-14239.

Ortiz de Montellano, P. R. 1986. Cytochrome P-450, structure, mechanism and biochemistry,. pp in: :"Cytochrome P-450." Plenum Press, New York.

Ostrowski, J., Barber, M. J., Rueger, D. C., Miller, B. E., Siegel, L. M., and Kredich, N. M. 1989. Characterization of the flavoprotein moieties of NADPH-Sulfite reductase from *Saklmonella typhimurium* and *Escherichia coli*. J. Biol. Chem. 264: 15796-15808.

Plapp, F. W. Jr. 1984. The genetic basis of insecticide resistance in the house fly: Evidence that a single locus plays a major role in metabolic resistance to insecticides. Pest. Biochem. Physiol. 22: 194-201.

Porter, T. D. and Kasper, C. B. 1985. Coding nucleotide sequence of rat NADPH-cytochrome P-450 oxidoreductase cDNA and identification of flavin-binding domains. Proc. Natl. Acad. Sci. USA 82: 973-977.

Porter, T. D. and Kasper, C. B. 1986. NADPH-cytochrome P-450 oxidoreductase: flavin mononucleotide and flavin adenine dinucleotide domains evolved from different flavoproteins. Bioch. 25: 1682-1687.

Poulos, T. L., Finzel, B. C., and Howard, A. J. 1987. High-resolution crystal structure of cytochrome P450cam. J. Mol. Biol. 195: 687-700.

Ray, J. W. 1965. Pest Infestation Research, p.59, HMSO, London.

Ronis, M. J. J., Hodgson, E., and Dauterman, W. C. 1988. Characterization of multiple forms of cytochrome P-450 from an insecticide resistant strain of house fly (*Musca domestica*). Pest. Biochem. Physiol. 32: 74-90.

Rose, H. A. 1985. The relationship between feeding specialization and host plants to aldrin epoxidase activities of midgut homogenates in larval lepidoptera. Ecol. Entomol. 10: 455-467.

Ruettinger, R. T., Wen, L. P., and Fulco, A. J. 1989. Coding nucleotide, 5' regulatory, and deduced amino acid sequences of P450bm-3, a single peptide cytochrome P-450:NADPH-P-450 reductase from *Bacillus megaterium*. J. Biol. Chem. 264: 10987-10995.

Shayiq, R. M. and Avadhani, N. G. 1989. Purification and characterization of a hepatic mitochondrial cytochrome P-450 active in aflatoxin B1 metabolism. Biochem. 28: 7546-7554.

Shen, A. L., Porter, T. D., Wilson, T. E., and Kasper, C. B. 1989. Structural analysis of the FMN binding domain of NADPH-cytochrome P-450 oxidoreductase by site-directed mutagenesis. J. Biol. Chem. 264: 7584-7589.

Shimizu, T., Hirano, K., Takahashi, M., Hatano, M., and Fuji-Kuriyama, Y. 1988. Site-directed mutageneses of rat liver cytochrome P-450d: axial ligand and heme incorporation. Biochem. 27: 4138-4141.

Shimizu, T., Sadeque, A. J. M., Hatano, M., and Fuji-Kuriyama, Y. 1989. Bindings of axial ligands to cytochrome P-450d mutants: a difference absorption spectral study. Biochem. Biophys. Acta 995: 116-121.

Stayton, P. S., Poulos, T. L., and Sligar, S. G. 1989. Putidaredoxin competitively inhibits cytochrome b5-cytochrome P-450cam association. Biochem. 28: 8201-8205.

Tsubaki, M., Iwamoto, Y., Hiwatashi, A., and Ichikawa, Y. 1989. Inhibition of electron transfer from adrenodoxin to cytochrome P-450scc by chemical modification with pyridoxal 5'-phosphate: identification of adrenodoxin-binding site of cytochrome P-450scc. Biochem. 28: 6899-6907.

Waters, L. C. and Nix, C. E. 1988. Regulation of insecticide resistance-related cytochrome P-450 expression in *Drosophila melanogaster*. Pest. Biochem. Physiol. 30: 214-227.

Wheelock, G. D. and Scott, J. G. 1989. Purification and initial characterization of the major cytochrome P-450 from a pyrethroid resistant house fly. pp in: "Molecular Insect Science." Hagedorn, H. H., Hildebrand, J. G., Kidwell, M. G. and Law, J. H., ed. Plenum, New York.

White, R. A., Franklin, R. T., and Agosin, M. 1979. Conversion of α-pinene to α-pinene oxide by rat liver and the bark beetle *Dendroctonus terebrans* microsomal fractions. Pest. Biochem. Physiol. 10: 233-242.

Yu, S. J. 1988. Microsomal S-demethylase activity in four lepidopterous insects. Pest. Biochem. Physiol. 31: 182-186.

ABSTRACTS

ω-AGATOXINS: A FAMILY OF NEURONAL CA^{++} CHANNEL ANTAGONISTS FROM FUNNEL WEB SPIDER (*Agelenopsis aperta*) VENOM

M.E. Adams,[1] V.P. Bindokas,[1] A.C. Dolphin,[3] J.S. Imperial,[2] B.M. Olivera,[2] J.M. Pocock,[1] R.H. Scott,[3] and V.J. Venema[1]

[1] Department of Entomology, University of California, Riverside, CA 92521
[2] Department of Biology, University of Utah, Salt Lake City, UT 84112
[3] Department of Pharmacology, St. Georges Hospital Medical School, London, SW17 ORE, U.K.

Five polypeptide antagonists (ω-agatoxins) of insect neuromuscular transmission were purified from *Agelenopsis aperta* spider venom. Physiological and biochemical data indicate that the ω-agatoxins comprise a family of presynaptic voltage-sensitive Ca^{++} channel antagonists. Each ω-agatoxin was isolated based on its ability to suppress transmitter release at the pre-pupal *Musca domestica* neuromuscular junction. Sequence analysis and binding experiments distinguish the ω-agatoxins as two subclasses. Type I toxins (ω-Aga-IA, ω-Aga-IB, ω-Aga-IC) are 7-8 kDa, have closely related N-terminal amino acid sequences, and exhibit characteristic tryptophan-like UV absorbance spectra. Complete Edman sequencing of ω-Aga-IA shows it to be a 66 amino acid polypeptide, including 9 cysteines and 5 tryptohan residues. Type II toxins (ω-Aga-IIA, ω-Aga-IIB) are 10-11 kDa, show low but significant sequence homology to Type I toxins, and exhibit mixed tryptophan- and tryosine-like absorbance. Type II, but not Type I toxins inhibit specific binding of ^{125}I-labeled ω-conotoxin GVIA (ω-Cg-Tx) to chick synaptosomal membranes. In accordance with these data, both Type II toxins and ω-CgTx block ^{45}Ca flux in chick synaptosomes, while Type I toxins have no effect.ω-agatoxins may define distinct Ca^{++} channel subtypes in both insects and vertebrates. For example, it appears that Type I and Type II toxins block different components of calcium dependent transmitter release from insect neuromuscular terminals. Calcium channels sensitive to ω-Aga-IA occur in both insect and frog skeletal muscle, in dorsal unpaired median neurons of the locust metathoracic ganglion, and in rat dorsal root ganglion neurons. Our binding data suggest that these actions involve a unique binding site unrelated to that defined by ω-CgTx. Inhibition of ω-CgTx binding by Type II toxins suggests that both toxins block Ca^{++} channels by a similar mechanism. Thus, the ω-agatoxins provide two novel sub-classes of polypeptide calcium channel antagonists active in diverse animal phyla. (Supported by NIH grant NS24472 and USDA grant 86-CRCR-1-2097.)

PURIFICATION AND CHARACTERIZATION OF TWO HEMOLYMPH PROTEINS FROM *Locusta migratoria*

J.B. Ancsin and G.R. Wyatt

Department of Biology, Queen's University, Kingston, Ontario, Canada

Two major hemolymph proteins of *Locusta migratoria* have been purified and their physical properties determined. They have distinctively different developmental profiles. Larval storage protein (LSP) occurs in larvae reaching a maximum concentration in the late fifth instar; it is found in lower amounts in early adults, decreasing to levels scarcely detectable by day 5. Storage protein (SP) is also present in larvae but after adult eclosion it remains a major component in adult hemolymph. Synthesis of SP in the adults is stimulated by a juvenile hormone analog. LSP gel filtration on Sephacryl-S 300 column indicates an apparent MW of 410 kDa. Its sedimentation coefficient is determined by sucrose density gradient centrifugation to be 15.5s. LSP is composed of up to 5 different subunits of 75 kDa. The pI-6.0 with microheterogeneity observed. Hexose content (D-glucose, D-mannose) is assayed at 10%. SP, with an apparent MW of 460 kDa with a sedimentation coefficient of 15.0s. It is composed

of 74 kDa and 77 kDa subunits. The pI-5.6 with some microheterogeneity observed. Hexose content was assayed at 7%. As with some holometabolous storage proteins, dissociation of SP is induced at moderately alkaline pH, which is reversible upon neutralization. No dissociation is observed for LSP at alkaline pH up to pH 9.6. The properties of these two proteins fit those of the "storage protein" class, hitherto studied chiefly in holometabolous species. Synthesis of such a protein in the adult stage is novel. (Supported by NSERC of Canada and U.S. NIH.)

Drosphila ACETYLCHOLINESTERASE: FINE STRUCTURE OF MOLECULAR FORMS IN WILD TYPE FLIES AND MUTANTS OF THE *Ace* LOCUS

M. Arpagaus,[1] and J.-P. Toutant[2]

[1] Pharmacology, University of Michigan, Ann Arbor
[2] Pharmacology, Case Western Reserve University, Cleveland, Ohio.

Acetylcholinesterase (AChE) terminates the action of ACh, a major neurotransmitter of the insect central nervous system. In adult *Drosophila*, AChE exists as a major membrane-bound dimer of globular subunits (Toutant *et al.*, 1988, J. Neurochem. 50:209-218) with the catalytic sites directed extracellularly (ectoenzyme). The association of each subunit with the membrane is mediated by a C-terminal glycolipid, (Fournier et al., 1988, J. Neurochem. 50:1158-1163) containing one alkyl and one acyl chain inserted in the outer leaflet of the lipid bilayer. Treatments by proteases or phosphatidylinositol-phospholipases C and D convert the membrane-bound dimeric AChE to a hydrophilic dimer with unaffected catalytic properties. During the development of *Drosophila*, we identified small amounts of a native hydrophobic monomer besides the major membrane-bound dimer. This monomer likely represents a precursor of the mature dimeric AChE (Arpagaus et al., 1988, Insect Biochem. 18:539-549; Toutant, 1989, Prog. Neurobiol. 32:423-446)

We investigated the structure of AChE in the following mutants of the *Ace* locus: j^{19}, j^{29}, j^{40}, j^{50} and lm^{38} (Hall et al., 1980, Genetics 96:939). Heterozygous or homozygous flies surviving at permissive temperatures, as well as animals with heteroallelic combinations, consistently showed a decrease of the level of AChE activity, but the enzyme was always present as a major glycolipid-anchored dimeric form. Therefore the mutations do not affect the assembly into dimers or the type of membrane association. In an attempt to further identify these mutations, we used the polymerase chain reaction to amplify a 550 bp-long segment of the coding region in the AChE gene containing the catalytic site. We are currently sequencing the amplified pieces.

PURIFICATION AND SEQUENCE ANALYSIS OF PHOTOAFFINITY-LABELED JUVENILE HORMONE RECEPTOR FROM THE EPIDERMAL NUCLEI OF *Manduca sexta*

J.K. Atkinson,[1] S.C. Towell,[2] M.A. Handley,[1] and G.D. Prestwich[1]

[1] Department of Chemistry, State University of New York, Stony Brook, New York 11794-3400
[2] Visiting Scientist from the Division of Entomology, CSIRO, Canberra, Australia

Three high specific activity, tritium-labeled photoaffinity labels have recently been employed in the specific, covalent modification of nuclear JH receptors from epidermal and fat body nuclei of early fifth instar larvae of the tobacco hornworm, *Manduca sexta* (S.R. Pailli, E.O. Osir, W.-s Eng, M.F. Boehm, M. Edwards, P. Kulcsár, I. Umjváry, K. Hiruma, G. D. Prestwich, and L. M. Riddiford, submitted). The first two, [^{3}H]-EHDA and [^{3}H]-EBDA (58

Ci/mmol), ar physiologically-active diazoacetate analogs of JH II and JH I, respectively. Photolabeling of proteins in epidermal cells (nuclei and cytosol), fat body, and hemolymph is selective and readily displaced by JH I or JH II. The second, [^{3}H]-MDK (80 Ci/mmol), is a diazomethyl ketone analog of methoprene, and selectively modifies the same 20kD protein found in nuclei of epidermal or fat body cells. Competitive displacement by methoprene and by JH I are pH dependent and somewhat more difficult to achieve. We report the purification of the 20kD protein after [^{3}H]-MDK photolabeling by ion exchange followed by either 1- or 2-dimensional SDS-PAGE. After transfer to an inert membrane, the electoblotted proteins are subjected to N-terminal sequence using gas-phase methods. This is a collaborative project with Professor Lynn M. Riddiford under an NSF Chemistry Life Processes Grant.

JUVENILE HORMONE AND ECDYSONE STIMULATION OF VITELLOGENIN PRODUCTION IN MALE HOUSEFLY FATBODY WITH RESPECT TO DIET

K.K. Bakke and T.S. Adams

Department of Zoology, North Dakota State University and Biosciences Research Laboratory USDA-ARS, Fargo, North Dakota, 58105

Male houseflies (Orlando strain) were held on a diet of either sugar or protein for 24 hours after emergence. The flies were then injected with 2.5 μg 20-hydroxyecdysone with or without 1 μg of the juvenile hormone analog methoprene. The flies were then placed on diets as follows (pre-treatment, post-treatment) sugar-sugar; protein-protein; sugar-protein; protein-sugar. 24 hours post injection the fatbodies were cultured and the media subsequently analyzed by rocket immuno-electrophoresis for vitellogenin. Flies fed protein for the duration of the experiment produced vitellogenin when given 20-hydroxyecdysone alone or in combination with methoprene (8.7 and 7.6 ng/μg fatbody, respectively). Sugar fed flies, or those with post-treatment sugar diet did not produce vitellogenin with 20-hydroxyecdysone treatment alone. When given in combination with methoprene however, vitellogenin production was observed (sugar-sugar: 11.0 ng/μg fatbody, protein-sugar: 11.6 ng/μg fatbody). Methoprene alone does not induce vitellogenin production in males. We conclude from these experiments that juvenile hormone can compensate for the lack of protein in the diet which is necessary for vitellogenin production.

GENE EXPRESSION IN MOSQUITO OOGENESIS

G.K. Banks

Department of Medical Parasitology, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT

Mosquito ovaries were dissected out before and at different time intervals after the blood meal. A profile of RNA synthesis was established based on the yields of two different extraction methods. Two peaks in RNA synthesis at 48 hours and 72 hours post blood meal were observed. The temporal pattern and nature of mRNA transcribed before and after the blood meal was examined by *in vitro* translation. Total translation products, reflecting mRNA transcription, followed the same temporal pattern of increase and decrease as total RNA. Analysis on electrophoresis produced no discernible differences in the pattern of bands present at each time interval, or in their intensity relative to each other, suggesting that the blood meal triggers a synchronous increase in transcription already ongoing in the unfed ovary. Analysis of *in vivo* proteins by electrophoresis was consistent with this interpretation. After the blood meal, a steady increase in total protein per ovary was observed, which occurred slightly later than the initial increase in total RNA. However the pattern of electrophoretic bands present, or their intensity relative to one another did not change, with the exception of a small number

of prominent bands which appear superimposed on the unfed ovary pattern at various time intervals post blood meal. These bands can be correlated to the uptake of vitellogenin and its conversion to vitellin.

ISOLATION, CHARACTERIZATION AND MOLECULAR BIOLOGY OF AN INSECT TRANSFERRIN

N.S. Bartfeld, and J.H. Law

Department of Biochemistry, University of Arizona, Tucson, Arizona, 85721

An 80 kilodaton iron-binding glycoprotein has been isolated from hemolymph of *Manduca sexta*. The protein binds iron both *in vivo* and *in vitro*, contains a high-mannose carbohydrate structure of the $man_9glcNAc_2$ type, and has a circular dichroic spectrum which indicates a secondary structure high in ß-sheet and low in α-helix. The protein is encoded by a 2.4 kilobase message which is present at high levels throughout the fourth and fifth larval instars, declines during the late wandering stage, then increases at pupation and is present in the adult. A 2.1 kilobase clone has been isolated by antibody screening of a λ ZAPII larval cDNA library and partially sequenced. The 5′ region of this clone encodes a protein whose N-terminal region has extensive homology to the second domain of both human and chicken transferrins. Several residues implicated in iron binding are also conserved in the insect protein. Based upon the similarities between the *M. sexta* protein and human serum transferrin with respect to secondary structure, sequence homology, and ability to bind ferric ion both *in vivo* and *in vitro*, we believe that this protein represents an insect transferrin, evolutionarily related to mammalian transferrins.

TWO TRANSLOCATION HETEROZYGOTES OF *Blattella germanica* ARRESTED EARLY IN DEVELOPMENT ARE DEFECTIVE IN PROTEOLYTIC PROCESSING OF VITELLIN

E.L. Beaudoin, X. Liu and J.H. Nordin

Department of Biochemistry, University of Massachusetts, Amherst, MA 01003

One goal of our research is to understand the mechanism that regulates vitellin (Vt) degradation during embryo development. Two translocation heterozygotes, T3/12 and T7/12 (THs), of *B. germanica* (Bg), produce mature oothecae in which 40-50% of the eggs are arrested at an early stage. Compared to eggs of embryos which develop normally, these eggs retain a lighter colored appearance, suggesting non-utilization of yolk (Ross, M., and Cochran D., 1975, J. Hered., 66:79; 1981, J. Hered., 72:57). We have studied yolk of individual TH eggs to ascertain the biochemical basis of the developmental arrest. In wild type eggs, average yolk granule size decreases greatly between days 4-5 postovulation, coincident with expression of proteases(s) and specific proteolytic processing of the total complement of Vt. In contrast, lighter colored eggs of both THs retain large YGs through day 6. A strong correlation also exists between the color of the TH's eggs at days 5-6 and extent of processing of their Vt as revealed by SDS-PAGE of yolk. Among 71 7/12 eggs, 77% of the darker eggs contained processed Vt and 88% of putatively arrested (lighter) egg's VT remained unprocessed or was only partially processed. Similar results have been obtained with 3/12 eggs. Yolk of these eggs has also been tested for protease activity with α N-benzyloxycarbonyl-L-lysine p-nitrophenyl ester. Average specific activity of 14 eggs with processed Vt (0.99 U/mg) was about 5 times that of 13 eggs with incompletely processed or unprocessed Vt (0.21 U/mg), with some of these eggs devoid of detectable activity. It is proposed that the protease(s) first

expressed in Bg yolk at day 4, functions to process Vt and that one major biochemical defect in arrested eggs of both THs is insufficient processing of Vt. Experiments are now in progress to characterize the protease(s) and to explore further the relationship between decrease in YG size and Vt utilization. (Supported by NSF DCB 88-19371.)

THE TOBACCO HORNWORM AS A MODEL FOR EXAMINATION OF MOLECULAR HOST-PARASITE RELATIONSHIPS

N.E. Beckage,[1,2,3] I. De Buron,[2] K.W. Schleifer,[2] S.H. Harwood [2]
K.S. Ammerman,[2] and D.J. Nesbit[2]

[1] USDA/ARS Stored Product Insects Research Unit
[2] Department of Entomology, University of Wisconsin, Madison, WI 53706
[3] Present address: Department of Entomology, University of California, Riverside, CA 92521

Manduca sexta serves as a useful model of parasitic infection and examination of the dynamics of host-parasite relationships. The braconid wasp *Cotesia congretata* develops in the hemocoel and secretes a variety of proteins during the later stages of its development. The parasite-derived teratocytes also secrete proteins *in vitro* and likely *in vivo*, indicating a dynamic protein exchange occurs between parasite and host. Although some biochemical changes appear to be mediated by the polydnavirus injected by the parasite into the host, others require the presence of developing parasites in conjunction with the virus for induction of the full spectrum of "normal" parasitic effects. Developmental arrest is not attributable to degeneration of the prothoracic glands, unlike results reported for other species, and arrest is mediated via more subtle mechanisms. The "early" and "late" stage parasitism-specific polypeptides are being characterized. Purification and sequencing of the 33 kDa "early" induced protein has been carried out. The native molecular weight of this protein appears to be 190 kDa and its N-terminal sequence is NH_2-Ser-Pro-Val-Lys-Thr-Ser-Leu-Gln-Glu-Arg-Val-Ile-Asp-X. These parasitism-induced proteins differ both in MW and isoelectric points from the antibacterial proteins of *Manduca sexta*. Ultrastructural and molecular characterization of the *Cotesia congregata* polydnavirus genome is now in progress. Prior to adult eclosion, two morphologically distinct types of virions are present in this species, a polynavirus and a long filmentous particle. Only the PDV virions are seen in the extracellular calyx fluid of adult female wasps. These particles have from 1 to 15 nucleocapsids packaged in a single envelope. The tobacco hornworm is also being utilized as a model for studying interactions with the nematode *Heterorhabditis heliothidis* and its associated pathogenic bacterium *Xenorhabdus luminescens*. Mechanisms of host pathology are being explored at the biochemical and ultrastructural levels. (Supported by the NSF, USDA, American Cancer Society, University of Wisconsin Graduate School, S.C. Johnson & Son, and a cooperative agreement between the University of Wisconsin and the USDA.)

ALLATINHIBIN, A NEUROHORMONAL INHIBITOR OF CORPUS ALLATUM ACTIVITY IN *Manduca sexta*

G. Bhaskaran, B.G. Unni, M.M. Pytel, K. Satyanarayana, and K.H. Dahm

Department of Biology, Texas A&M University, College Station, TX 77843-3258

The Corpora Allata (CA) of last instar larvae of *Manduca sexta* become incapable of synthesizing juvenile hormone early in the wandering stage. This is due to the loss of juvenile hormone acid methyltransferase activity in CA; however, CA continue to synthesize juvenile hormone acid. The change in CA activity is induced by an inhibitory factor from the brain,

allatinhibin (AI). AI-inhibited CA are unable to cause a larval molt when transplanted into allatectomized fourth instar larvae (bioassay). Active CA from day 0 Vth instar larvae became inhibited when co-incubated *in vitro* with brains from wandering larvae. These findings led to the development of an *in vitro* - *in vivo* assay for AI. Brains were first incubated in tissue culture medium. Then CA were incubated in the brain-free medium and bioassayed. Maximal AI activity was obtained from brains of wandering (day 4) larvae. AI activity was absent in the medium in which day 0 Vth instar or pupal brains were incubated. Dose-response data for day 4 Vth instar brain medium showed that AI is active at 0.03 brain equivalents/200 μl medium. AI is heat-labile, protease sensitive, has a molecular size between 1.0-3.5 kDa, and is clearly distinct from allatostatins described by other investigators.

MOSQUITO P-450 GENES AND PYRETHROID INSECTICIDE RESISTANCE

R.G. Bonet, J. Crampton, and H. Townson

Wolfson Unit of Molecular Genetics, Department of Medical Entomology, Liverpool School of Tropical Medicine, Liverpool L3 5QA, United Kingdom

Cytochrome P-450s comprise a multigene family of haem-containing proteins, some of which, by virtue of their monoxygenase activity, play an important role in detoxification of xenobiotics in insects, including insecticides. We have used an 18-mer degenerate oligonucleotide, homologous to the conserved sequence of haem binding site of several phenobarbital inducible P-450s from the rat and rabbit, to identify and isolate putative P-450 genes from the mosquito *Aedes aegypti*. Following screening of a cosmid genomic library, twenty positive clones were lifted for detailed analysis. One clone of 35.1 kb showed evidence of two adjacent P-450-like sequences flanked by repetitive elements. A sub-clone in pUC12 of one of these sequences, p450s63, shows homology with a number of published sequences for cytochrome P-450s.

We have used the synthetic oligonucleotide and the p450s63 clone as probes to examine the P-450 genes of pyrethroid insecticide sensitive and resistant strains of the malaria vector *Anopheles stephensi*. Northern blot analysis show evidence of elevated levels of a P-450 transcript in the pyrethroid resistant strain. This suggests that the P-450 mediated resistance seen in this strain is due to enhanced expression rather than a change in substrate specificity of the P-450 concerned.

BIOSYNTHESIS OF THE "ESSENTIAL" FATTY ACID, LINOLEIC ACID, IN THE AMERICAN COCKROACH, *Periplaneta americana*

C.E. Borgeson,[1] T.S. Kellermeyer,[1] G.J. Blomquist,[1] T.J. Kurtti,[2] and U.G. Munderloh[2]

[1] Department of Biochemistry, University of Nevada, Reno, NV 89557-00014
[2] Department of Entomology, University of Minnesota, Minneapolis, MN 55108

Linoleic acid, 18:2(n-6), a dietary requirement in most animals, has been shown to be synthesized in several insect species. To determine that the synthesis occurs in insect tissue and is not due to contaminating microorganisms, various tissues from the American cockroach and from the house cricket were incubated with radiolabeled substrates under sterile conditions. Radio-HPLC analysis of tissues from both insects demonstrated *de novo* synthesis of linoleic acid. Since no contaminating bacteria or fungi were present in the media and since

synthesis occurred in the cricket tissues, which do not contain bacteriocytes, as well as in the cockroach tissues, which do, the synthesis of linoleic acid is attributed to insect tissue.

The requirement for dietary linoleic acid in most animals is due to the absence of Δ^{12} desaturase, the enzyme responsible for the desaturation of oleic acid, 18:1(n-9), to 18:2(n-6). Data will be presented characterizing this enzyme in microsomal preparations from *P. americana*. Experiments were designed to determine whether it is similar to the plant Δ^{12} desaturase or to other animal desaturases, and whether it is the same in all the species of insects in which it occurs. The oleic acid substrate for the cockroach Δ^{12} desaturase was determined to be the acyl-CoA form. This is in contrast to the plant Δ^{12} desaturase, which desaturates 18:1(n-9) complexed to phosphatidylcholine or phosphatidylenthanolamine.

A CHIRAL SPECIFIC ANTISERUM FOR JUVENILE HORMONE

D.W. Borst, D. Hunnicutt, and Y.C. Toong

Department of Biological Sciences, Illinois State University, Normal IL 61761, and School of Chemistry, University of Sains Malaysia, Penang, Malaysia

Current methods for quantifying juvenile hormone (JH), such as radioimmunoassay (RIA) and gas chromatography/mass spectroscopy, are cumbersome, requiring extensive sample preparation and derivatization. One factor that may limit the performance of current RIAs for JH may be the use of racemic JH as a hapten. Recently, 10*R*-JH III has been purified from a plant source (Toong et al., 1988, Nature 333:170). We used this material as a hapten to generate an antiserum against this naturally-occurring enantiomer of JH. 10*R*-JH III was converted to its acid by treatment with methanol:1N NaOH (1:1), and then coupled to thyroglobulin *via* the N-hydroxysuccinimidyl ester (>100 haptens/thyroglobulin). Rabbits were injected and boosted with the antigen (in Freund's adjuvant). The antiserum was collected and characterized using racemic [^{3}H]JH III (NEN-Dupont) as the ligand. The antiserum specifically bound JH III, showed 50% displacement of ligand at 3 pg/tube. JH I and JH II were 7% and 31%, respectively, as effective as racemic JH III. JH III preparations that were 92% chirally-pure were 140% and 25% (for 10*R* and 10*S*, respectively) as active as racemic JH III. Methyl farnesoate, JH III acid, and JH III diol did not interact with the antibody. These data indicate that the affinity of the antiserum for 10*R*-JH III is at least 10-fold > than that for 10*S* enantiomer. The high sensitivity and selectivity of this antiserum suggest that it will be useful in the detection and quantification of JHs from biological samples. (Supported by USDA grant 86-CRCR-1-1064.)

SYNTHESIS AND DEGRADATION OF JH III IN THE MOSQUITO *Aedes aegypti*

D. Borovsky, D.A. Carlson, I. Ujary, and G.D. Prestwich

University of Florida-IFAS, Florida Medical Entomology Laboratory, Vero Beach, FL, 32962, USDA, SEA AR IAMARL, Gainesville, FL 32604, and Department of Chemistry, State University of New York, Stony Brook, NY 11794-3400

The biosynthesis of JH III *in vivo* and *in vitro* was followed with [^{3}H]methyl farnesoate at different times after adult eclosion and after the blood meal. Newly synthesized [^{3}H]JH III was chromatographed on HPLC and further analyzed using preparative GC. Following the blood meal an initial increase in the synthesis of JH III was observed both *in vivo* and *in vitro*, followed with a decrease in the synthesis to a level found in sugar fed females. Between 10 h and 96 h after the blood meal the synthesis of JH III increased reaching a peak at 96 h and

declined thereafter. JH III metabolism was followed using HPLC showed that the ratio between JH III metabolites: diol-acid/acid/diol was 25/4/1. These results indicate that JH III is metabolized first to the acid and then immediately to the diol-acid by epoxide hydrase which hydrates JH III 24 times slower than JH III acid. (Supported by USDA CRCR 1-2394 to D.B. and NSF grants DCB-8509629 and DCB-8812322 to G.D.P.)

MOSQUITO OOSTATIC HORMONE: A PEPTIDE OF VIRAL ORIGIN

D. Borovsky, and D.A. Carlson

University of Florida-IFAS, Florida Medical Entomology Laboratory, Vero Beach, FL 32962
USDA, SEA AR IAMARL, Gainesville, FL 32604

Mosquito oostatic hormone has been purified from the ovaries of *Aedes aegypti* using low and high pressure liquid chromatography. The hormone has been sequenced using Tandem Quadruple Mass Spectrometry with Ion Cyclotron Resonance. The hormone is a decapeptide of M_r 1,047 which exhibited 70% homology to the HIV II. Several analogs were synthesized using solid phase peptide synthesizer. Injection of the natural hormone and the decapeptide analogs into mosquitoes, sand flies, cat fleas, house and dog flies inhibited proteolytic enzyme biosynthesis and food digestion in the midgut. Although egg development was also inhibited in mosquitoes and sand flies the primary target tissue of the hormone appears to be the midgut epithelial cells and not the ovary or the brain endocrine system. A relationship between hormone synthesis and regulation of trypsinlike synthesis in the midgut is proposed. (Supported by USDA CRCR 1-2394 to D.B.)

CORTICAL GRANULES OF THE MARINE SHRIMP, *Penaeus vannamei*

J.Y. Bradfield, S.M. Rankin, and L.L. Keeley

Department of Entomology, Texas A&M University, College Station, TX

Cortical granules (CGs, a.k.a. cortical rods, cortical bodies) are membrane-bound organelles in oöcytes of many animal (e.g. echinoderms, annelids, amphibians, placental mammals). Typically, rod-like or spherical CGs are assembled during oöcyte development and become associated with the oölemma in the mature egg. In response to one or more stimuli (such as contact with water at spawning, or fertilization), CGs are rapidly extruded to form a layer of material that encompasses the egg. In some cases, the CG-derived layer is retained throughout embryonic development; in other species, such as shrimp, the layer rapidly dissipates in the surrounding seawater. CG composition, function, and regulation are largely unknown. It is speculated that they prevent polyspermy or serve as an environmental protectant.

The club-shaped CGs of the favored shrimp of mariculture, *Penaeus vannamei*, are extraordinarily prominent, constituting 15-20% of the volume of the mature oöcyte. To examine the function and regulation of these organelles, we have generated a cloned cDNA and monospecific antibody for a major CG polypeptide. CG mRNA is a large transcript (6.5 kb) found in the *P. vannamei* ovary but not in other tissues. the corresponding 205 kDa polypeptide appears to be immunologically unrelated to other shrimp proteins. The CG polypeptide is absent in early oöcytes, and accumulates massively during development to represent 5-10% of total ovarian protein at the time of spawning. After the spawn,, CGs are apparently lost rapidly from fertilized eggs, becoming immunologically undetectable at the multicellular gastrula stage. cDNA probes and antibodies are begin used to study the regulation and function of the Cgs which are an important component of reproduction in this decapod crustacean and other animals.

DNA ELEMENTS AND PROTEIN FACTORS THAT MAY REGULATE LOCUST VITELLOGENIN GENE EXPRESSION

R.P. Braun, and G.R. Wyatt

Department of Biology, Queen's University, Kingston, Ontario

Proteins which specifically recognize DNA sequences 5′ to the vitellogenin genes (A and B) of the African migratory locust are being studied. These genes are regulated by juvenile hormone and provide an attractive model to study DNA elements and their corresponding transcription factors involved in JH-stimulated transcription. There is no assay at present to measure transcriptional activity of the native or modified promoters in this system. Rather, to identify elements potentially involved in transcription, a gel retardation assay is being used, which can detect proteins that recognize specific DNA sequences. Profiles of the proteins from different tissues of both sexes at different stages of development, which bind specifically to vitellogenin gene-associated DNA should identify those proteins crucial for proper expression of these genes. Both nuclear and whole cell extracts from adult female fat body have been employed and binding conditions have been optimized for a number of proteins. Their precise binding sites are being determined and evidence for their functional significance is being sought. (Supported by NSERC of Canada and U.S. NIH)

MOLECULAR ELEMENTS OF OLFACTORY SIGNAL TRANSDUCTION IN INSECT ANTENNAE

H. Breer, I. Boekhoff, K. Raming, and J. Strotmann

University of Hohenheim, Institute of Zoophysiology, Garbenstr. 30, D-7000 Stuttgart 70, FRG

The ability to perceive chemicals in their surroundings is common to most organisms and insects are especially known to have an enormous ability to process chemical signals. As yet, little is known about the molecular mechanisms of olfactory reception and the signal transduction cascade in insect antennae. It has been suggested that upon binding of odorants to specific receptors a common transductory reaction cascade is triggered, mediated by specific G-proteins. Based on the molecular size, the specificity of bacterial toxin catalyzed ADP-ribosylation and the immunoreactivity with antibodies of designed specificity, subunits of G-proteins have been identified in antennae of various species.

As the phosphatidylinositol-system has been proposed to play a key role in visual signal transduction of invertebrates, the activity of phospholipase C, the key enzyme of this system, has been determined in antennae of various insects. The antennal high specific activity of the enzyme was sensitive to Ca^{2+}-ions and GTP and was found to be significantly stimulated by hydrophobic odorants in a GTP-dependent manner. This observation may be considered as a first indication of a functional involvement of the phosphatidylinositol-system in olfactory signal transduction of insects.

Towards an identification of putative odorant-receptors, the hybridoma technique in combination with a selective screening procedure was used to raise monoclonal antibodies which selectively recognize proteins of antennal membranes. These antibodies were applied to identify the membrane proteins in Western blots, to localize the antigens topochemically in antennal cells and to isolate the proteins by immunoaffinity chromatography.

MOLECULAR BIOLOGY OF CHOLINERGIC SYNAPSES IN INSECTS

H. Breer, M. Knipper, J. Krieger, and E. Tarelius

University of Hohenheim, Institute of Zoophysiology, Garbenstr. 30, D-7000 Stuttgart 7000, FRG

Preparations from the highly cholinergic nervous tissue of insects have been used to study molecular elements of cholinergic synapses. The high affinity uptake of choline, which is supposed to be the rate limiting regulatory step for acetylcholine synthesis has been explored concerning its energetic and regulation in approaches using isolated nerve terminals and synaptosomal ghosts. The transport rate appears to be controlled via protein kinases. Hemicholinium-3 as well as specific monoclonal antibodies have been employed to identify molecular elements of this carrier system. The transporter protein has been purified and functionally reconstituted in liposomes; it represents a glycosylated membrane protein. Subtypes of muscarinic receptors for acetylcholine have been detected in the nervous system of insects. Whereas nerve cell somata contain preferentially M_1-type receptors, M_2-receptors are located at nerve terminals, they appear to be negatively coupled to adenylate cyclase and are obviously involved in the negative feedback regulation of acetylcholine release. The nicotinic acetylcholine receptor type, which is blocked by α-bungarotoxin predominates in insect nervous tissue. The receptor protein has been isolated and purified to homogeneity and appears to be an oligomeric complex of identical or very similar polypeptides. Low molecular weight compounds have been isolated that may be considered as endogenous ligands for α-toxin bindings sites. Furthermore a 20 Kd polypeptide has been identified which appears to be involved in regulating the number of active receptors in the membrane via mechanisms controlled by protein kinase A. The channel properties of this neuronal nicotinic acetylcholine receptor has been elucidated after reconstituting the purified receptor protein into planar lipid bilayers, they closely resemble the *Torpedo* receptor; they show higher conductance and shorter open times than the extrasynaptic ACh-receptors of insect perikarya.

LOCALIZATION OF Aea-HP-I AND VERTEBRATE-LIKE PEPTIDES IN THE NEUROENDOCRINE AND MIDGUT ENDOCRINE SYSTEMS OF *Aedes aegypti*

M.R. Brown,[1] J.W. Crim,[2] and A.O. Lea[1]

Department of Entomology[1] and Zoology,[2] University of Georgia, Athens, GA 30602

After characterizing the structure of decapeptide, aedes head peptide I (Aea-HP-I), isolated from mosquito heads, we produced a polyclonal antiserum to the synthetic peptide. This antiserum and antisera for the vertebrate peptides, dynorphin, Leu-enkephalin, and urotensin I, were used for immunocytochemistry to detect immunoreactive factors in the nervous system and midgut of female mosquitoes.

Leu-enkephalin- and urotensin I-like immunoreactivity were observed in specific neurosecretory cells and their axons and were not found in other neurons in the female brain. In contrast, dynorphin-like immunoreactivity was observed only in non-neurosecretory neurons. Aea-HP-I was present in neurosecretory cells, other neurons and midgut endocrine cells. Urotensin I-like immunoreactivity also was detected in midgut endocrine cells, whereas Leu-enkephalin and dynorphin immunoreactivity were not. The discovery of Aea-HP-I and urotensin I-like immunoreactivity in both the neuroendocrine and midgut endocrine systems confirms the existence of a brain-midgut endocrine axis in the mosquito which is comparable to that of vertebrates. (Supported by NIH AI-17297 to AOL.)

METHYL PARAOXON-RESISTANT ACETYLCHOLINESTERASE CONTROLLED BY A SINGLE GENE IN THE TOBACCO BUDWORM, *Heliothis virescens*

T.M. Brown, and P.K. Bryson

Department of Entomology, Clemson University, Clemson, SC 29634-0365

Larvae and adults of a strain of *Heliothis virescens* from Woodrow, South Carolina carry a high level of resistance to methyl parathion without selection in the laboratory. Resistance is correlated to reduced sensitivity of acetylcholinesterase to methyl paraoxon inhibition in preparations from both larval ganglia and from adult heads. Segregation of acetylcholinesterase insensitivity was demonstrated in several backcrosses to hybrids made with a sensitive strain from Florence, South Carolina which was started from a single pair. The resistant enzyme is approximately 10-fold less sensitive to methyl paraoxon and it is completely insensitive to propoxur. Kinetics of the substrate reaction and the inhibition reaction for both resistant and susceptible enzymes will be described. RR and RS genotypes are discriminated by relative sensitivities to other organophosphorus compounds and by reactivation from methyl(phenyl) phosphinylation. Genetic linkage of this trait to a marker locus has been demonstrated (Heckel et al., these proceedings).

CLONING OF RICKETTSIA-LIKE MICROORGANISMS FROM THE TSETSE FLY

P. Burke, J. Crampton, and H. Townson

Wolfson Unit of Molecular Genetics, Department of Medical Entomology, Liverpool School of Tropical Medicine, Liverpool L3 5QA, United Kingdom

Rickettsia-like microorganisms (RLMOs) have been implicated in mating incompatibility in mosquitoes belong to the *Culex pipiens* and *Aedes scutellaris* groups. They have also been found to influence susceptibility to trypanosomes in tsetse flies.

We have constructed a phage library using DNA of the RLMO from the tsetse *Glossina morsitans* and by differential screening selected clones which can be used to identify RLMOs in both tsetse and mosquito tissue.

Sequence date for one such clone confirms that it is of prokarotic origin. Hybridization experiments suggest some sequence divergence between the homologous sequences of the RLMO from tsetse and those from mosquitoes.

Such cloned sequences could be used to investigate the, as yet, largely unknown mechanisms by which the microorganisms influence their host biology.

THE REGULATION OF DIURESIS IN ADULT *Heliothis zea*: *in vivo* AND *in vitro* STUDIES

D.W. Bushman, and J.O. Nelson

Department of Entomology, University of Maryland, College Park, MD 20742

Adult *Heliothis zea* exhibit periods of diuresis following emergence from the pupa and later in adult life following feeding. *In vivo* assay systems have been used to investigate the regulation of these diuretic responses. Soluble factors present in the brain and abdominal ganglia stimulate diuresis as measured by fluid weight loss in a post-eclosion assay. Diuresis following intrahaemocoelic injection of saline is also stimulated by homogenates of brain or

abdominal ganglia. Preliminary studies with *Manduca sexta* suggest that there is considerable cross-reactivity associated with the diuretic factors of these two species.

Results from a post-feeding assay indicate diuresis following feeding is significantly reduced by an antidiuretic factor present in the corpora cardiaca/corpora allata complex. The frontal ganglion exerts neural control over crop emptying, thus influencing the rate at which fluid ultimately enters the haemolymph for subsequent excretion following eclosion and feeding.

The role of cAMP and several biogenic amines was investigated both *in vivo* and *in vitro*. Serotonin, dopamine, and octopamine were tested in and *in vivo* post injection diuresis assay. Fluid weight loss was not affected by these amines at the concentrations tested. Moreover, cAMP had no effect on diuresis in the same assay. An *in vitro* assay that measures cAMP production by Malpighian tubules was also used to evaluate the role of the biogenic amines and the diuretic factor from the brain and abdominal ganglia. The results indicated that cAMP is not involved in the signal transduction of the diuretic factor in *H. zea* Malpighian tubules.

CARBOHYDRATES AND AMINO ACIDS COLLECTED FROM THE PHLOEM SAP OF POINSETTIA AND PUMPKIN AND FROM THE HONEYDEW OF *Bemisia tabaci*

D.N. Byrne and W.B. Miller

Departments of Entomology and Plant Sciences, University of Arizona, Tucson, AZ 85721

To find clues to why a strain of the sweet potato whitefly, *Bemisia tabaci* (Genn.), from Florida is able to so readily adopt new host plants, we examined the phoem sap of pumpkin and poinsettia and the honeydew from *B. tabaci* feeding on these two plants. We specifically examined their amino acid and carbohydrate contents and the mass of the honeydew produced. The most obvious reason this strain is successful is that it processes more phloem sap and therefore has access to more of the relatively rare amino acids and vitamins found there. There were 15 different amino acids in the phloem sap of poinsettia and 14 in the phloem sap of pumpkin. Concentrations of most amino acids were reduced in the honeydew when compared to levels found in phloem sap. Some of these are likely used in protein synthesis, others are used in the production of the six additional amino acids found in the honeydew which are not found in phloem sap. Carbohydrates from phloem sap were not unusual (in poinsettia we found sucrose, glucose and fructose; in pumpkin we found these plus raffinose, stachyose and galactose). In the honeydew of poinsettia we found the trisaccharide melezitose and in the honeydew of pumpkin we found large quantities of what is possibly a sugar new to Insecta, trehalulose. There were large quantities of glutamine in honeydew. Because we found very little purines there, glutamine may be the form in which excess nitrogen is excreted.

BIOCHEMICAL STUDIES OF ELEVATED ESTERASE ELECTROMORPHS IN AN ITALIAN STRAIN OF *Culex pipiens*

A. Callaghan

Department of Medical Parasitology, London School of Hygiene and Tropical Medicine, Keppel Street, London, WCIE 7HT

An organophosphate-resistant strain of *Culex pipiens* from Italy was found to contain at least eight non-specific esterases, each capable of displaying elevated or non-elevated esterase activity. Particular combinations of these esterases were isolated in different strains by selective breeding. Inhibition studies identified three of the elevated esterases (A2, A4 and B2) as carboxylesterases. Biochemical analysis showed that the three esterases (A1, A2 and A4, had isoelectric points (pI's) of between 5.6-6.5, whilst B2 had a pI of 5.6. Molecular weight determination on polyacrylamide gradient gels, revealed that A and B esterases differed

greatly, with A esterases having a molecular weight of approximately 110 kDa and B esterases having a molecular weight of approximately 65 kDa. The results were consistent with a hypothesis put forward by other workers, that A esterases exist as dimers and B esterases exist as monomers. A and B esterases were highly thermolabile, losing up to 50% activity at 4 °C over an hour. Some evidence was found to suggest that there were differences in thermolabiltiy between the A esterases, which could be a pre-determining factor in their selection by insecticides at different latitudes. Partial purification succeeded in separating A esterases from B esterases, due to the loss of B esterase activity. Kinetic studies indicated that A1, A2 and A4 had the same K_m for the substrates α-naphthyl acetate and α-naphthyl butyrate. It is suggested that esterase polymorphism is of little importance with respect to actual resistance conferred, the selection of an isoenzyme being determined by the prevailing esterase frequencies prior to insecticide use. Of far more importance is the vast potential for resistance that this polymorphism appears to convey.

BIOSYNTHESIS OF *Musca domestica* LIPOPHORIN

M. De L. Capurro and A.G. De Bianchi

Department of Biochemistry, University of Sao Paulo, Sao Paulo 01498, CP 20790, Brazil

In *M. domestica*, lipids are transported via hemolymph by lipophorin, a high density lipoprotein (HDL). In *manduca sexta* it was demonstrated that lipophorin is synthesized by fat bodies and secreted to hemolymph as a lipoprotein containing only ApoLp I, ApoLp II and phospholipids. This newly synthesized particle is loaded with diacylglycerol and other lipids by midgut cells. (Sarvamangala et al., J. Biol. Chem. 261:17174, 1986).

The incubation of fat bodies of *M. domestica* feeding larvae *in vitro* with [^{35}S]-methionine or [^{32}P]-orthophosphate shows that the lipophorin synthesized and secreted to the incubation medium has a little higher density than the mature larval lipophorin. On the other hand, incubation of *M. domestica* fat bodies obtained from feeding larvae with purified lipophorin shows that the fat bodies take lipids of this protein resulting in particles more dense than the feeding larvae mature lipophorin. According to these results there are two possible explanations for the lipophorin biosynthesis in *M. domestica*. The lipophorin is synthesized depleted of lipids and immediately after secretion it is loaded with lipids by the fat bodies or the lipophorin is synthesized and partially loaded with lipids before the secretion to incubation medium occurs.

Anyway, the *M. domestica* lipophorin apparently does not necessarily need to be loaded with lipids by midgut cells. (Supported by grants from FAFESP, FINEP and CNPq.)

BIOSYNTHETIC INCORPORATION OF JUVENILE HORMONE III AND FARNESYL COMPOUNDS INTO CANTHARIDIN

J.E. Carrel, J.P. McCormick, J.H. Brill, and M. del D. Mustiga

Division of Biological Sciences and Department of Chemistry, University of Missouri-Columbia, MO 65211

Cantharidin, a potent defensive substance in blister beetles, is derived from farnesol and molecular oxygen by unknown metabolic processes that are consistent with involvement of JH III in the pathway. Using radiolabeled and deuterated farnesyl compounds as substrates, we found little if any incorporation of JH III into cantharidin but we detected large amounts of several farnesyl compounds were incorporated into this substance. We conclude that it is unlikely that JH III is significantly involved as a substrate in the cantharidin biosynthetic

pathway. We wish to acknowledge the support of the NIH and the gift of labeled JH III from Dr. David Schooley and Glenn Prestwich.

BACULOVIRUS *Autographa californica* NUCLEAR POLYHEDROSIS VIRUS (AcMNPV)

D.D. Carson, M.D. Summers, and L.A. Guarino

Department of Entomology, Texas A&M University, College Station, TX 77843

The AcMNPV gene, IE-N, has been functionally mapped in the viral genome by transient expression assays in *Spodoptera frugiperda* cell culture. By its temporal expression during the viral life cycle and its transient expression in the absence of viral protein translation, IE-N is an immediate early gene. IE-N mRNA is highly expressed 0-3, and undetectable after 6-8, hours post infection. To further study the regulation and function of IE-N, the nucleotide sequence of 2 kb of the PstI-N genomic fragment of AcMNPV encoding IE-N was determined and was analyzed for possible viral regulatory motifs. Subsequently, IE-N-CAT (NCAT) constructs were analyzed by transient expression assays to determine both cis- and trans-acting viral factors influencing the expression of the IE-N transcription unit. The AcMNPV enhancer, hrl, when cis-linked to NCAT stimulated expression in the presence of host cellular factors alone. Functional IE-N further stimulated the hrl-NCAT construct as well as the NCAT alone. This suggested that IE-N is auto-regulated in an enhancer independent fashion. Down regulation by another immediate early AcMNPV gene, IE-1 is also shown to be mediated only by hr1 sequences. Thus, transient expression assays have been utilized to determine viral factors which may be involved in gene regulation of the AcMNPV gene, IE-N, during early viral infection.

IMMUNE RESPONSES OF MOSQUITOES TO FILARIAE AND BACTERIA

U. Chaithong and H. Townson

Department of Medical Entomology, Liverpool School of Tropical Medicine, Liverpool L3 5QA, United Kingdom

By means of *in vivo* haemolymph transfer experiments, we have demonstrated that haemolymph from the mosquito *Aedes aegypti*, immunized with *Brugia pahangi* (Nematoda: Filaroidea), *E. coli*, a β 1,3-glucan (laminarin) and bacterial lipopolysaccharide (LPS), is partially protective against subsequent infection with the filaria. By SDS-PAGE electrophoresis we have identified a number of haemolymph proteins inducible by these treatments, including some whose molecular weights suggest homology with the previously described immune proteins attacins, lysozyme, diptericin, and ceropins. A related poster by Knapp and Crampton describes the cloning and characterization of a number of sequences from *A. aegypti* homologous to know immune protein genes.

Which, if any, of these inducible proteins are responsible for the protective effects of the transferred haemolymph against filarial nematodes is as yet unclear, indeed several of them are believed to have specificity for microbial cell wall components. We have also examined some of the changes in haemocytes in response to filariae, bacteria, laminarin and LPS. These results will be discussed in relation to the emerging picture of the spectrum of immune response produced in mosquitoes.

INVESTIGATE THE NATURE OF VIRAL PERSISTENT INFECTIONS USING HZ-1 BACULOVIRUS AS THE MODEL SYSTEM

Y.C. Chao, H.A. Wood, and C.Y. Jang

Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan, Republic of China and Boyce Thompson Institute for Plant Research, Cornell University, Ithaca, New York

Hz-1 virus, a non-occluded insect baculovirus, is one of the best model systems to study viral persistent infection. We have conducted experiments to find the viral gene(s) responsible for this phenomenon. As the first step toward this goal, we restriction mapped its 228.3 kb double-stranded circular genome using the restriction enzymes *Hin*dIII, *Xho*I, *Eco*RI, *Sst*II, and *Sma*I. Deletion maps of this virus were also derived from deletion clones. Hz-1 deletion maps showed that there is a primary site for deletions to occur. Viral specific transcripts were then mapped and found surprisingly that although there were more than 100 viral specific transcripts detected during productive infection, there was only one transcript detected during persistent infection. This transcript is constitutively expressed regardless of age and type of the infected cells. Our data suggests that this transcript is encoded by a unique early gene. The possible mechanism of the expression regulation of this gene will be discussed.

CHANGES IN DISTRIBUTION OF ACTIN MICROFILAMENTS IN A LEPIDOPTERAN CELL LINE DURING INFECTION WITH A BACULOVIRUS

C.A. Charlton and L.E. Volkman

Department of Entomology, University of California, Berkeley, CA

Proper assembly of nucleocapsids is prevented by the presence of 5 μg/ml cytochalasin D (CD) during replication of the baculovirus, *Autographa californica* nuclear polyhedrosis virus (AcMNPV), suggesting a role for actin microfilaments in this process. A fluorescence microscopy study was conducted to examine changes in distribution of microfilaments and to correlate changing patterns with events in the virus life cycle. Uninfected *Spodoptera frugiperda* cells show rhodamine-conjugated phalloidin staining of fine filaments in the cytoplasm. From one to three hours post infection (p. i.), at the time of the virus uptake, coarse, cycloheximide-insensitive fibers are seen extending from spots on the plasma membrane into the cytoplasm. Staining of viral capsid protein shows that virus particles are located near to or along these fibers at this time. Beginning at 5 hours p. i., large bundles of cycloheximide-sensitive but aphidicolin-insensitive microfilaments appear on the ventral surface of the cell. These bundles are almost always in cells which have rounded up and precede expression of capsid protein in the cell. Replication of viral DNA takes place in a virogenic stroma in the nucleus. At 12 hours p. i., capsid protein localizes to the stroma, while microfilament staining is restricted to a few spots at the edges of the stroma, but still within the nucleus. As nucleocapsids assemble, they move from the stroma to the nuclear membrane and then to the plasma membrane where they bud from the cell. During this time of active virus budding, microfilaments (aphidicolin-sensitive) fill the area between the virogenic stroma and the nuclear membrane where they colocalize with viral nucleocapsids. In the presence of CD, actin does not polymerize in the nucleus and aberrant nucleocapsids are formed.

BIOSYNTHESIS AND ENDOCRINE REGULATION OF THE FEMALE SEX PHEROMONE OF THE GERMAN COCKROACH, *Blattella germanica*

J. Chase, G.J. Blomquist, and C. Schal

Department of Biochemistry, University of Nevada Reno, Reno, NY 89557-0014 and Entomology, Rutgers University, New Brunswick, NJ 08903

The structural relationships between the cuticular hydrocarbons and the contact sex pheromone of the German cockroach, *blattella germanica*, and the precursors of the hydrocarbons, were investigated by radiotracer, GC-MS, HPLC and ^{13}C NMR techniques. The ketone fraction, which includes the major contact sex pheromone component, was analyzed by GC-MS before and after reduction to hydrocarbon. Females have the 3,11- and 3,9-dimethyl C_{27} and C_{29} alkanes, but only the 3,11-isomer of the dimethyl ketones. Evidence indicates that the production of the contact sex pheromone results from the sex specific oxidation of 3,11-dimethylalkanes to pheromone components by the female. Incorporation of the amino acids [G-^{3}H]valine, [4,5-^{3}H]isoleucine, and [3,4-^{14}C]methionine, as well as [1,4-^{14}C]- and [2,3 ^{14}C]succinates into hydrocarbon indicates that these precursors serve as the methyl branch group donor. Carbon-13 NMR studies of methyl branched cuticular hydrocarbons and the female contact sex pheromone indicate that the methyl branching groups of the 3,x-dimethyl alkanes are inserted early in chain elongation. Data show that the highest rate of pheromone production occurs with 8-day-old females, and that juvenile hormone plays a major role in regulating sex pheromone production.

ISOLATION AND CHARACTERIZATION OF A LYSOSOMAL ENZYME, CATHEPSIN D, FROM THE MOSQUITO *Aedes aegypti*

W.-L. Cho and A.S. Raikhel

Program in Genetics and Department of Entomology, Michigan State University, East Lansing, MI 48824

Lyosomes play an important role in termination of vitellogenin secretion from the mosquito fat body. As a basis for studying regulation of this lysosomal activity at the molecular level, a lysosomal enzyme, cathepsin D, was purified and characterized. The protocol for its isolation involved ammonium sulfate (30%-50%) and acid (pH 2.5) precipitations of protein extracts from whole previtellogenic mosquitoes followed by cation exchange chromatography. Purity of the enzyme was monitored by SDS-PAGE and silver staining of the gels. The native molecular weight of the purified enzyme as determined by polyacrylamide gel electrophoresis under non-denaturing conditions was 80,000. SDS-PAGE resolved the enzyme into a single polypeptide with Mr=40,000 suggesting that it exists as a dimer in its non-denatured state. The pI of the purified enzyme was 5.5 as determined by isoelectrofocusing gel electrophoresis. The purified enzyme exhibits properties characteristic of cathepsin D. It utilizes hemoglobin as a substrate and its activity is completely inhibited by pepstatin-A and 6 M urea but not by 10 mM KCN. Optimal activity of the purified mosquito cathepsin D was obtained at pH 3.0 and 45°C. (Supported by grants from NIH and MSU Biotechnology Center.)

NEUROENDOCRINE REGULATION OF DIURESIS IN LARVAE OF THE TOBACCO BUDWORM, *Heliothis virescens*

L.L. Chyli, J.S. Chung, and T.K. Hayes

Laboratories for Invertebrate Neuroendocrine Research, Department of Entomology, Texas Agriculture Experiment Station, Texas A&M University, College Station, TX 77843

Diuretic factors were studied in the central nervous system of larvae of the tobacco budworm Heliothis virescens, using [^{14}C]urea as a sensitive indicator for water movement through Malpighian tubules *in vitro*. The assay required the presence of NA^+ and a pH of 6.0-6.2 for maximum activity. Fluid transport by the Malpighian tubules is insensitive to the Na^+/K^+ ATPase inhibitor ouabain, but sensitive to ethacrynic acid, a Na^+ channel blocker, and to furosemide, a Na^+/Cl^- cotransport blocker. The rate of fluid secretion was low in Malpighian tubules of fourth instar larvae, but increased by >300% during a 24 hour period of intense feeding in the fifth instar (Stage $_1$). The larval body weight also increased by more than 3-fold, to its final mature weight, during this same time. Diuretic activity declined to a minimum after feeding ceased during the burrowing-digging stage that preceded pupation. Malpighian tubules from 24-hr starved, fifth instar larvae were more responsive to extracts of nervous tissues than were tubules from feeding larvae. Diuretic activity was distributed throughout all parts of the larval central nervous system with the brain having the greatest activity. Diuretic activity was present in brains from third, fourth, and fifth instar larvae and from pupae and pharate adults. In dose-response studies, 0.3 to 10 brain equivalents caused a linear increase of over 300% in fluid secretion rate by *in vitro* Malpighian tubules. *In vivo*, brian extracts doubled the rate of dye clearance from the hemolymph. Diuretic activity in nervous tissue extracts was stable to boiling but sensitive to trypsin, chymotrypsin and pyrogluamate aminopeptidase but not to aminopeptidase M. Fluid secretion by *in vitro* tubules was increased to a maximum of 35-40% by dbcAMP in a dose-dependent manner, but brain extracts were more effective. Phorbol esters elevated fluid transport and acted synergistically with brain extracts. Isolation studies with extracts of heads from pharate adults demonstrated the presence of both a diuretic factor and an antidiuretic factor that suppressed fluid movement through isolated larvae Malpighian tubules. The *Heliothis* diuretic factor is highly hydrophobic. (Supported by the Robert J. Kleberg, Jr., and Helen C. Kleberg Foundation.)

A CYTOCHROME P-450 cDNA CLONE FROM *Drosophila melanogaster*

B.J. Cochrane, P.C. de Belligny, F. Holtsberg, J. Pursey, and R. Morton

Department of Biology, University of S. Florida, Tampa, FL and
Department of Biology, Hamilton University, Hamilton, Ontario, Canada

Selection for malathion resistance in *Drosophila melanogaster* results in the elevation of the level of a particular cytochrome P-450, suggesting that this protein may be directly involved in insecticide metabolism (Houpt et al., 1988, Genome, 30:844-853). This protein was partially purified by hydrophobic and ion exchange chromatography, and a polyclonal antiserum prepared and purified by removal of immunoglobulins specific for microsomal proteins expressed in sensitive flies. This antibody was employed to screen a λgt11 expression library for clones producing crossreactive fusion protein. From 80,000 plaques, a single clone, designated λDmP450-1, was obtained, which crosshybridizes with a P-450 specific cDNA clone from house flies (Feyereisen et al., 1989, Proc. Nat'l. Acad. Sci. 86:1465-1569). This clone, of ca. 750 bp, was employed as a probe to obtain a clone of 1.8 kb from a λgt10 cDNA library prepared from adult male mRNA. This clone hybridizes to one genomic sequence, as well as two mRNA species of 1.8 and 5 kb. The former is detectable in all fly strains, while the latter is only seen in resistant ones. Analysis of genomic Southern blots indicates the presence of

one homologous sequence in the *Drosophila* genome. Data will be presented on the inferred sequence of the protein coded for this clone, as well as the cytological location of the gene(s) in question. (Supported by the American Cancer Society, Florida Division.)

ISOLATION AND CHARACTERIZATION OF A GLUTATHIONE S-TRANSFERASE cDNA FROM *Drosophila melanogaster*

B.J. Cochrane, J.J. Morrissey, and J. Philips

Department of Biology, University of South Florida, Tampa, FL, and Department of Molecular Biology and Genetics, Guelph University, Guelph, Ontario, Canada

In *Drosophila* there is a single isoform of glutathione S-transferase (GST) that accounts for more than 90% of the total activity towards 1-chloro 2,4-sinitrobenzene in adults, and consists of two immunologically crossreactive subunits of 35 kDa and 28.5 kDa. Antisera directed against the protein were prepared and employed in the screening of an adult cDNA library in the expression vector λgt11. A single clone was isolated that, based upon immunological criteria, was inferred to code for the small subunit of GST. This clone hybridizes strongly to an mRNA of 1.3 kb that is present in both third instar larvae and adults, and weakly to one of 2.3 kb. *In situ* hybridization reveals that the clone hybridizes to a single sit, 87CD, in the right arm of the chromosome III. To date, sequence analysis has revealed no homology with any other reported GST sequences. The complete nucleotide sequence will be presented, as well as the effects of heterozygosity for deficiencies in the 87CD region on enzyme activity and subunit abundance.

DEVELOPMENT OF IMPROVED INSECT TRANSFORMATION TECHNIQUES

A.F. Cockburn

Insects Affecting Man and Animals Research Laboratory, USDA, Gainesville, FL 32604

The *Drosophila* P element transformation procedure has been ineffective in other insects. We are developing general techniques that can be used in transformation of insects and that will allow much larger numbers of transformants to be generated in *Drosophila*.

We have developed a novel method for mass-injection insect embryos which eliminates the need for injection of individual embryos. The effectiveness of this technique for introduction of DNA into *Drosophila* embryos is being evaluated by transformation. A practical mass-injection technique will eliminate the major obstacle to insect transformation-the difficulty in introducing DNA into embryos.

Very active transposons (analogous to P elements) are hard to identify except by genetic crosses that are difficult to perform in most insects. We have a developed a method for screening for potentially active transposons by identifying repetitive DNA sequences that are discontinuously distributed in closely related species. This approach has been tried in the *Anopheles quadrimaculatus* species complex, and three repetitive sequences have been identified that are present in one species of the complex and absent in others.

PRELIMINARY CHARACTERIZATION AND KINETICS OF SALIVARY AND MIDGUT PROTEINASES OF A PREDATORY HETEROPTERAN

A.C. Cohen

Honey Bee Biology and Biological Control of Insects, USDA-ARS, 2000 East Allen Road, Tucson, AZ 85719

Proteinases from a predatory reduviid salivary gland complex (SGC), anterior midgut (AMG) and posterior midgut (PMG) were studied with hemoglobin, BAPNA and LPNA (artificial protein substrates) used as substrates. Temperature and pH optima were determined, and kinetics measured with various proteinase inhibitors used to characterize the enzymes. The SGC proved to be the most active site of endoproteinase activity while the AMG was most active with respect to exoproteinase secretion.

GLUCOSE DEHYDROGENASE: EVOLUTIONARY CONSERVATION OF A MOLTING FLUID ENZYME IN DIPTERA AND LEPIDOPTERA

D.L. Cox-Foster, K. Golden, and M. Briggs

Department of Entomology, University Park, PA 16802

The glucose dehydrogenase (GLD) gene is evolutionally conserved between the Diptera and Lepidoptera. In *Drosophila melanogaster*, the enzyme is essential for formation of the operculum and successful eclosion of the adult fly. The Gld gene is expresses prior to each molt in *Drosophila* in correlation with increased ecdysone titer, by the epidermis or epidermal derivatives (Cavener, et al., 1986, EMBO J. 4:2939-2948; Cox-Foster, et al., 1989, unpublished). This gene is conserved in *Manduca sexta* as evidenced by homology with probes derived from the *Drosophila* Gld gene on both southern and northern filters under conditions of high stringency. In *Manduca*, the enzyme is expressed likewise at each molt by epidermal cell; evidence also suggests that the enzyme is activated in the molting fluid and not in the epidermal cells. In molting fluid isolated from pharate adults, the enzyme has a pI of 4.2 and is also a glycoprotein like the *Drosophila* enzyme. This enzyme has also been detected during molts in other insects ranging from the primitive hemimetabolous orders to other higher holometabolous orders. This expression of the enzyme and the molecular conservation between the Diptera and Lepidoptera suggests that GLD serves a functionally conserved role in the insect molt.

PERIODICITY GENES IN THE MOSQUITO, *Aedes aegypti*

J. Crampton, S. Cullen, and T. Knapp

Wolfson Unit of Molecular Genetics, Liverpool School of Tropical Medicine, Liverpool L3 5QA, United Kingdom

Genes involved in controlling physiological processes may provide interesting targets for gene manipulation. One gene of particular interest is the periodicity or *per* gene, because of its involvement in controlling rhythmic processes in many organisms including insects. In *Drosophila melanogaster* this gene controls the timing of eclosion and some rhythmic components of courtship song. We describe the cloning of this gene from *Aedes aegypti* using a synthetic oligonucleotide probe to screen a genomic clone bank representative of the *A.*

aegypti genome. The probe used was based on the conserved portion of the *per* gene or *per*-related sequences described from a number of different organisms. The characterization and sequence of this gene in *A. aegypti* is described. We eventually hope to be able to analyse the types of behavioral processes which this gene controls in mosquitoes and may therefore be able to assess whether the manipulation of this gene has potential for future control strategies.

THE RIBOSOMAL GENES OF THE MOSQUITO, *Aedes aegypti*

J. Crampton and K. Gale

Wolfson Unit of Molecular Genetics, Liverpool School of Tropical Medicine, Liverpool L3 5QA, U.K.

The characterization of the ribosomal genes of the mosquito, *Aedes aegypti* is described. The rDNA repeat of *A. aegypti* is 9.0 kb in length and individual rDNA repeats exhibit a high degree of homogeneity with respect to length and the position of restriction enzyme recognition sites within the rDNA. Preliminary mapping data together with partial digestion experiments demonstrate that, as in all other higher eukaryotes, the rDNA repeats are arranged in a head-to-tail, tandemly repeating manner.

The restriction mapping of cloned rDNA repeats confirms the largely uniform length of the *A. aegypti* rDNA repeat and has provided a more detailed physical map of the DNA. Transcription mapping has allowed the allocation of identities to the various restriction fragments and the approximate positioning of the transcription unit. The estimate of rDNA repeat copy number in *A. aegypti* (approximately 500 copies per haploid genome) is similar to the estimate reported for the closely relates species *Aedes albopictus*, of 430 copies per genome. Analysis of the variation of the rDNA repeat unit both within individual mosquitoes and between strains of *A. aegypti*, has served to confirm the remarkable homogeneity of the rDNA in this insect.

GUT MOTILITY OF *Heliothis zea* CONTROLLED BY FMRF-AMIDE-RELATED PEPTIDES

J.W. Crim,[1] A.C. Jenkins,[1] M.R. Brown,[2] and A.O. Lea[2]

Department of Zoology[1] and Entomology,[2] University of Georgia, Athens, GA 30602

Axons of the musculature of the posterior midgut of the corn earworm, *Heliothis zea*, exhibit immunoreactivity related to Phe-Met-Arg-Phe-amide (FMRFa; molluscan cardioactive peptide). We have used an *in vitro* assay to examine effects of selected peptides of the RF-amide family on muscle contractions of the gut. Isolated midgut/hindgut preparations of 5th instar larvae were perfused continuously with *Heliothis* saline, and isometric contractions were measured with a force displacement transducer (FT-O3C) connected to a Grass polygraph. Gut preparations exhibited robust, spontaneous myogenic activity, particularly of the pylorus. Activity was stimulated by FMRFa or serotonin (5-HT), mainly as dose-related increases in contraction frequency. Contractility was inhibited potently by *Aedes aegypti* Head Peptide I (Aea-HP-I; pERPhPSLKTRFa) or Leucomyosupressin (pEDVHVFLRFa), through dose-related reductions of both contraction amplitude and frequency. Unfractionated hemolymph was markedly stimulatory. Treatment of *H. zea* larvae *in vivo* by injections of Aea-HP-I reduced frass production and feeding. Collectively, these observations suggest that endogenous neuropeptides and peptide hormones of the RF-amide family may regulate motility of the gut of *H. zea*. (Supported by USDA 88-37252-3972 to JWC & AOL and NIH AI-17297 to AOL.)

ALTERATION OF JUVENILE HORMONE ESTERASE ACTIVITY BY PARASITOID-DERIVED FACTORS

D.L. Dahlman, D. Zhang, and T.J. Neary

Department of Entomology, University of Kentucky, Lexington, KY 40546

Teratocytes (extra-embryonic cells derived from the serosal membrane of the egg of certain internal parasitoids) from *Microplitis croceipes* placed into nonparasitized *Heliothis virescens* larvae cause developmental changes in the host similar to those observed in larvae parasitized by *M. croceipes*. Changes included delayed larval mortality, incomplete larval-pupal ecdysis and delayed pupation. Responses were dose dependent and younger teratocytes caused a more pronounced response. Fat body tissues were particularly affected. Juvenile hormone esterase (JHE) activity was dramatically depressed when the normal number of teratocytes from a single parasitoid (750) were injected but even 350 cells caused a significant reduction in JHE activity. *H. virescens* hemocytes, *Cotesia congregata* teratocytes, unfertilized *M. croceipes* eggs and cell walls of *Micrococcus* all failed to inhibit JHE activity nor was the developmental rate of the larvae affected by these treatments. *H. virescens* growth and development as well as JHE activity also were inhibited by injections of the egg-free calyx fluid from *M. croceipes* which contained polydnavirus but not poison gland components. Polydnaviruses from another parasitoid-host complex have been shown to infect host hemocytes and DNA from male parasitoids hybridizes with labeled probes prepared from polydnavirus. It is possible that the teratocytes contain the virus or at least the genomic information and thus, the polydnavirus may be responsible for the observed effects on the *H. virescens* larvae. The separation of these two important components of potential influence is the target of continued investigation. (Supported in part by USDA Competitive Grant 85-CRCR-1-1764 and by R.J. Reynolds.)

DEVELOPMENT OF RESISTANCE TO THE 72kDa TOXIN OF *Bacillus thuringiensis israelensis* IN *Culex quinquefasciatus*

S.-M. Dai and S.S. Gill

Department of Entomology, University of California, Riverside, CA 92521

Numerous *B. thruingiensis* strains have been isolated which show activity towards lepidopteran, dipteran or colepteran insects. Most of these *B. thuringiensis* strains produce a proteinaceous parasporal body during sporulation which is usually toxin to insect larvae upon ingestion. Utilization of *B. thuringiensis* for insect control is likely to face major problems with insects resistance in the near future. In fact cases of isolated resistance have been reported with at least two lepidopteran insects, *Plodia interpunctella* and *Heliothis virescens*. Further we have shown that it is possible to rapidly develop resistance in the mosquito, *C. quinquefasciatus* to the 72 kDa toxin of *B. t. israelensis*. Selection for resistance was performed with highly purified 72 kDa toxin crystals and fourth instar *C. quiquefasciatus* larvae. The larvae which were used in the selection were exposed tot he toxin for 24 hr and the mortality recorded for 24 and 48 hr. The selected and unselected larvae were then evaluated for the level of resistance. After 20 generations the Lc_{50} values for the selected strain was 70-80 fold greater than that for the susceptible colony. In addition to the development of resistance to the 72 kDa toxin it was observed that this resistant strain was also less susceptible to the whole intact parasporal body. (Supported by NIEHS grant ES03298 at the University of California Mosquito Control Program Grant No. 89-07.)

CLONING GENES THAT ARE ACTIVATED DURING POSTEMBRYONIC NEUROGENESIS IN *Manduca sexta*

M.-T. B. Davis

USDA, ARS, Insect Chemical Ecology Laboratory and Insect Hormone Laboratory, B-467, BARC-East, Beltsville, MD 20705

Different from hemimetabolous insects, most of the adult brain in holometabolous insects develops during postembryonic neurogenesis (Panov, 1963, Rev. Ent. URSS, 42:337-350). Since postembryonic neurogenesis in Lepidoptera is a critical period of neuroblast differentiation, neuronal cell death, and synaptogenesis for transformation of larval neurons into adult neurons (Nordlander and Edwards, 1969, Wilhem Roux' Aide. 162:197-217; Booker and Truman, 1987, J. Comp. Neurol. 225:548-559), I have been interested in studying expression of brain proteins and their genes during this developmental period in *Manduca sexta*. By improving the O'Farrell two-dimensional gel electrophoresis, a single gel was capable of resolving complex mixtures of *Manduca* brain polypeptides in which molecular weights range from less than 14,000 daltons to more than 95,000 daltons. From these gel separations, I have identified 21 intracellular brain polypeptides and 65 putative neurosecretory polypeptides that change in concentration during *Manduca* adult brain development. From *Manduca* brain cDNA library constructed in lambda Zap phage, I have also cloned six genes that are differentially expressed during postembryonic neurogenesis. The expression of three of these genes is brain-specific. Activation of the cloned genes during this period suggests that the controlled expression of these genes is important for postembryonic neurogenesis and brain differentiation.

A VASOPRESSIN-LIKE IMMUNOREACTIVE NEUROHEMAL SYSTEM IN LEPIDOPTERA

N.T. Davis and H. Lehman

Arizona Research Laboratories, Division of Neurobiology, University of Arizona, Tucson, AZ 85721

Using an antibody to lys-vaspression, vasopressin-like immunoreactive (VLI) material can be demonstrated in the corpora cardiaca and in lateral pairs of cells in the second through last abdominal ganglia of *Lymantria dispar* and *Manduca sexta*. Similar sets of VLI neurons are found in the abdominal ganglia of other insects, and the VLI cells appear to be distinctly different from the vasopressin-immunoreactive neurons previously noted in insects.

The VLI neurons of the abdominal ganglia project by way of the ventral nerves to the perivisceral organs which appear to provide for neurohemal release of the VLI material. These VLI neurons are identical to one set of neurons that have been shown to produce bursicon, indicating that bursicon and the VLI material may be colocalized in these cells. Similarly, VLI staining of the cells of the CC may indicate colocalization of the VLI material with AKH.

Intensity of the staining of the VLI material increases with thirst and/or starvation and decreases following feeding and forced libation. This appears to indicate the release of VLI material is associated with feeding and/or diuresis.

This VLI neurohemal system is found in all postembryonic stages; it is inconspicuous in the prepupa and staining increases in the pharate adult. A competitive ELISA method was used to quantitate this change. The results indicate that the CNS of the late pharate adult of *M. sexta* contains about 112 pmoles of VLI material and that the amount of this material in the brain complex doubles in the final days of development of the pharate adult.

A LARVAL SPECIFIC LIPOPROTEIN IN *Musca domestica*

A.G. De Bianchi and M. de L. Capurro

Department of Biochemistry, University of São Paulo, São Paulo 01498, CP 20780 Brazil

During the purification of larval *M. domestica* lipophorin a contaminant lipoprotein floats together with it in a potassium bromide density gradient. This protein is separated from lipophorin in a glycerol gradient ultracentrifugation. This lipoprotein must have a higher molecular weight than lipophorin since it goes to the bottom of the glycerol gradient centrifugation tubes ahead of lipophorin. This lipoprotein does not occur in adults thus it was denominated larval lipoprotein (LLP). The LLP has a density equal to 1.134 ± 0.007g/ml and is composed of at least five types of apolipoproteins (ApoLLP I to ApoLLP V) with molecular weights equal to 48,000 ± 2,500, 41,000 ± 2,100, 26,900 ± 600, 24,500 ± 600 and 21,899 ± 600.

Preliminary data suggests that LLP lipidic moiety is composed mainly of diacylglycerols (1,2 and 1,3) and cholesterol. Phospholipids and hydrocarbons are also present but in minor relative amounts. Fat bodies from feeding larvae incubated *in vitro* with [^{35}S]-methionine synthesizes LLP that is secreted to the incubation medium. The newly synthesized LLP has the same density as mature LLP. This lipoprotein resembles some mammalian lipoproteins due to the fact that it is composed by several low molecular weight apolipoproteins. However, the lipidic moiety is completely different in mammalian and larval insect lipoprotein. (Supported by grants from FAPESP, FINEP, and CNPq.)

RESISTANCE TO THE IMMUNE RESPONSE OF *Lygus lineolaris* BY THE PARASITOID *Leiophron uniformis*

J.W. Debolt and J.L. Flexner

Honey Bee Biology and Biological Control of Insects Research, USDA-ARS, 2000 E. Allen Road, Tucson, AZ 85719

Lygus lineolaris (Heteroptera:Miridae) effectively protect themselves from the parasitoid *Leiophron uniformis* (Hymenoptera:Braconidae) by encapsulating and killing the wasp's eggs. Recently an encapsulation resistant biotype of the parasitoid has been discovered. Studies into the nature of the wasp's resistance mechanism indicate that unlike species which inject immunosuppressing polydna viruses, resistant *L. uniformis* do not suppress the immune response of the host when they oviposit. This suggests that the surface properties of resistant eggs prevent their recognition as non-self by the host. In another parasitoid species, virus-like particles coating the surface of its eggs prevent their recognition as non-self. However, preliminary evidence from studies utilizing crosses between resistant and susceptible *L. uniformis* strains indicate that resistance may not be viral.

HEREDITARY VIRUSES OF *Heliothis*?: CHROMATIN ASSOCIATED VIRUS-LIKE PARTICLES IN TESTES OF THREE SPECIES OF *Heliothis*, *Heliocoverpa assulta*, AND INTERSPECIFIC HYBRIDS OF THE ABOVE

M.E. DeGrugillier

Biosciences Research Laboratory, USDA, ARS, Fargo, ND 58105

Virus-like particles, (VLP's) were found associated with the chromatin of spermatocyst cell nuclei in adult *Heliothis virescens*, *H. subflexa*, *H. zea*, and *Heliocoverpa assulta*. In

addition, the 50-55nm spherical particles were present in spermatocyst and follicle cell nuclei of testes of F_1 males derived from interspecific crosses involving *H. virescens* x *H. subflexa* and *H. assulta* x *H. zea*. The VLP's were also found in testes of 150th generation backcross (BC) males that were derived from the hybridization of *H. virescens* x *H. subflexa*. The chromatin-associated VLP'S suggests the presence of a hereditary virus and this virus may prove to be the basis for the sterility observed in BC males.

A MOLECULAR MARKER FOR INSECT EPITHELIAL MORPHOGENESIS

J.L. Denburg, and B.A. Norbeck

Biology Department, University of Iowa, Iowa City, IA 52242

Morphogenetic movements occurring within epithelial cell layers play a central role in the production of the general body plan of multicellular organisms. Depending on their position in the embryo and the time of development, cell will undergo specific changes in their properties that will generate the formation of new structures. A molecular marker has been identified that is specific for cell directly involved in epithelial morphogenesis. A monoclonal antibody has been developed that selectively binds to epithelial cells that are undergoing any of three very different morphogenetic movement- invagination, evagination and epibody. Neighboring cells not involved in these developmental processes are not labelled by the antibody. The antigen is transiently present on the cells for a period just prior to and during the morphogenetic activity. It is localized predominantly on the apical surface of the cells and is associated with the cytoskeleton. The spatial, temporal and subcellular distributions of antibody binding during development indicates a role for the antigen in epithelial morphogenesis.

POST-TRANSLATIONAL PROCESSING OF MOSQUITO VITELLOGENIN

T.S. Dhadialla, and A.S. Raikhel

Department of Entomology and Program in Cell and Molecular Biology, Michigan State University, East Lansing, MI 48824

Vitellogenin (Vg), the hemolymph precursor to the major egg yolk protein, is synthesized in the fat body of blood-fed female mosquito *Aedes aegypti*. Previously, we demonstrated that the mosquito Vg consists of subunits (Mr=200,000 and 66,000), both of which arise from a common 250 kDa precursor which is glycosylated and phosphorylated. In this report we present data on the post-translational processing of mosquito Vg subunits. *In vitro* pulse-chase experiments revealed rapid proteolytic cleavage of the 250 kDa precursor to two polypeptides with Mr=190,000 and 62,000. Incubation of fat bodies with tunicamycin *in vitro* inhibited glycosylation, phosphorylation and secretion but not the cleavage of the primary product of translation of Vg mRNA, hence resulting in the accumulation of two polypeptides with Mr=185,000 and 49,000. Treatment of secreted Vg with Endo-H or alkaline phosphatase revealed that glycosylation accounted for 10 kDa and 13 kDa for the large and small Vg subunits, respectively, while phosphorylation accounted 7 kDa and 4 kDa for the same subunits. Further pulse-chase experiments showed that transformation of the cleavage products into mature Vg subunits occurred as the last step prior to secretion of Vg. This step was inhibited by an ionophore, monensin, and therefore occurred in the Golgi complex. Sulfation

as an additional, previously unknown, modification of mosquito Vg was revealed by the incorporation of sodium[^{35}S]sulfate into both subunits. Since sulfation of Vg was predominantly blocked by monenisn, the final maturation of Vg subunits in the Golgi complex is, at least in part, due to this modification. (Supported by NIH research grant AI-24716.)

INSECT IMMUNITY. MOLECULAR CHARACTERIZATION OF DIPTERICINS AND INSECT DEFENSINS, TWO FAMILIES OF INDUCED ANTIBACTERIAL PEPTIDES FROM THE DIPTERAN *Phormia terranovae*

J.-L. Dimarcq, J.-M Reichhart, J. Lambert, C. Wicker, J. Hoffmann, and D. Hoffmann

Laboratorie de Biologie Générale, UA 672 du C.N.R.S. "Endocrinologie et Immunologie des Insectes", 12 rue de l'Univeristé 67000 Strasbourg, France

We have isolated and characterized diptericins and insect defensins, two novel families of antibacterial peptides from immune blood of larvae of *Phormia terranovae* and established their peptide sequence by Edman degradation. Using oligonucleotide probes, we have screened cDNA libraries from immune fat body and obtained various clones from which the peptide sequence of the precursor molecules could be deduced. We have expressed the diptericins and defensins in yeast; the recombinant defensins have full biological activity and we will report on the activity spectrum of these molecules. The presentation will include preliminary data on related molecules in *Drosophila*.

DIVERSIFICATION OF YOLK PROTEINS: AN INSECT EMBRYONIC STRATEGY?

R.E. Dompenciel-Rivera, J.G. Kunkel and D.E. Leonard

Program in Molecular and Cellular Biology and Department of Entomology, University of Massachusetts, Amherst, MA 01003

Egg production in insects is generally considered to require the availability of a yolk precursor, vitellogenin. However, investigators have shown that artificially activated vitellogenin-deficient *Bombyx mori* eggs matured in male hosts and proceeded with embryonic and post-embryonic development. In the lepidopteran *Lymantria dispar*, behavioral and developmental characteristics of the newly hatched larvae may be correlated with egg quality. The first eggs deposited by females are larger than subsequently produced, resulting in unequal partitioning of the resources. In order to study the distribution of yolk proteins, titers were determined from oocytes along ovarioles and eggs at different developmental stages. A stepladder pattern of increasing concentration from basal to distal chorionated oocytes was found for vitellin and arylphorin in contrast to slow, steady decrease in kopophorin concentration. An additional egg specific protein was also isolated and found to show a slow steady increase. During embryonic development, vitellin is not used rapidly and persists at considerable levels in the newly hatched larvae. This carry-over should serve as a nutritional resource for the first instar larvae before feeding takes place. A different kind of yolk protein has been shown to be deposited in considerable amounts in the yolk spheres of oocytes in other lepidopteran. A similar protein is present in *L. dispar* eggs which seems to play an important role during embryogenesis because of its complete disappearance before diapause. (Supported by USDA grants to DL & JGK and OMGSR Fellowship to RED.)

HOMEOBOX GENES IN THE MOSQUITO, *Aedes aegypti*

P. Eggleston

Wolfson Unit of Molecular Genetics, Liverpool School of Tropical Medicine, Liverpool L3 5QA, United Kindom

We have begun the isolation and characterization of developmental control genes in the mosquito, *Aedes aegypti.* This project forms part of our overall strategy for the identification of potential target genes for use in novel insect control programs. Larval genomic DNA was used to construct a library in the phage vector lambda gt.WES. A number of sequences have now been isolated from this library following hybridization to a synthetic oligonucleotide probe which corresponds to the highly conserved region of the *Antennapaedia*-like homeobox of *Drosophila melanogaster.* We anticipate that at least some of these sequences will exert a controlling influence over segment identity, segmentation and pattern formation in this mosquito. On the basis of cross hybridization and restriction digest patterns, the sequences fall into nine discrete classes. Representatives of two of these have been subcloned into plasmid vectors for further analysis. These clones, designated pUChbox 4A and pUChbox17A have been mapped in detail using restriction endonucleases and the regions around the homeobox are currently being sequenced. Analysis of the sequence data will clarify the nature of the developmental control genes involved. Subsequently, we intend to investigate the spatial and temporal expression of these sequences in sectioned and whole mount embryos.

SEX PHEROMONE BIOSYNTHETIC PATHWAY IN *Spodoptera littoralis*

G. Fabrias, and T. Martinez

Department of Biological Organic Chemistry, Centro de Investigaciön y Desarrollo, C.S.I.C., Barcelona, Spain. Present address: Department of Entomology, University of Arizona, Tucson, AZ 85721

Deuterium-labeled fatty acids have been used to elucidate the sex pheromone biosynthetic pathway in *Spodoptera littoralis*. Label from palmitic acid was incorporated into all pheromone acetates and their corresponding fatty acyl precursors. (Z,E)-9,11-tetradecadienyl acetate, the major component of the pheromone blend, is synthesized from palmitic acid *via* tetradecanoic acid, which by the action of a specific (E)-11 desaturase and subsequently a (Z)-9 desaturase is converted into (Z,E)-9,11-tetradecadienoate. By further reduction and acetylation this compound leads to the diene acetate.

Labeled precursors applied to the pheromone gland during the photophase were also incorporated into the pheromone. Percentage of labeled (Z,E)-9,11-tetradecadienyl acetate relative to natural compound was significantly higher during the light period as compared to the scotophase.

Label incorporation from several fatty acid precursors into the pheromone was stimulated by injection of brain-subesophageal ganglion extract during the photophase.

IDENTIFICATION OF p74, A VIRAL CODED GENE PRODUCT ESSENTIAL FOR VIRULENCE OF BACULOVIRUS OCCLUSION BODIES IN INSECTS

P. Faulkner,[1] J. Kuzio,[1] and R. Jaques[2]

[1] Department of Microbiology and Immunology, Queen's University, Kingston, Ontario, Canada K7L 3N6
[2] Agriculture Canada Research Station, Harrow, Ontario, Canada, NOR 1GO

DNA sequencing of the *Hin*dIII-P fragment of the baculovirus *Autographa californica* nuclear polyhedrosis virus downstream of a major late protein, p10, revealed the presence of an open reading frame (ORF) 1935 nucleotides in length and in opposite polarity to p10. The gene product is considered essential for virus replication in *Trichoplusia ni* larvae since infection with occlusion bodies from a mutant, Ac228z, in which portions of adjacent carboxy termini from peptides p74 and p10 were deleted, failed to kill larvae, whereas virus with deletions in p10 alone were as infectious to larvae as wild type virus. The ORF has the potential to code for a polypeptide of 645 amino acid residues (M,73, 819) and was designated p74. Time course analysis of RNA synthesis in infected cells suggested that the gene's promoter was weak and was most active at 16-20 post-infection. The transcription initiation site of the RNA was located to a sequence (TATTG) which is dissimilar to a consensus sequence (ATAAG) found near the transcription start site of other late baculovirus genes. The p74 gene was cloned into a baculovirus expression vector and a recombinant virus overexpressed the p74 protein.

IDENTIFICATION OF A CELL LINE VESICLE PROMOTING FACTOR IN LARVAL TISSUES OF *Trichoplusia ni*

S.M. Ferkovich, H. Oberlander, C. Dillard, and E. Leach

Insect Attractants, Behavior & Basic Biology Research Laboratory, USDA, ARS, P.O. Box 14565, Gainesville, FL 32604

The hormone-responsive cell line, IAL-TND1, was derived from imaginal wing discs of the cabbage looper moth, *Trichoplusia ni.* The cells originally grew as multicellular vesicles but after a year in culture the cells underwent a morphological change to an aggregate form. Reversal to vesicle form could be induced by adding larval hemolymph to the aggregate cultures. Likewise, inhibition of vesicle formation could be effected by the addition of physiological levels of 20-hydroxyecdysone. The vesicle promoting activity of larval hemolymph is due to a non-glycosylated, polypeptide (VPF) with an estimated molecular weight of 16.9 kDa on SDS gels. The amino-terminal amino acid sequence for VPF has been determined and a polyclonal antibody to VPF has be raised. Of the total hemolymph protein, the VPF comprised 0.75% and was active in the VPF bioassay at 10-6 M. VPF was detected by immunoblotting in larval wing discs, fat body, CNS, and hemocytes and was localized in the wing discs and on the cellular surfaces of vesicles by light microscopy immunofluorescence using a goat anti-rabbit fluorescence-conjugated IgG.

IMMUNOHISTOCHEMICAL ANALYSIS OF ADIPOKINETIC HORMONES (AKHs) AND THEIR PRECURSOR RELATED PEPTIDES (APRPs) IN THE BRAIN AND NEUROENDOCRINE COMPLEX OF THE LOCUST *Schistocerca gregaria*

J. Fischer-Lougheed, and M. O'Shea

Laboratorie de Neurobiologie, Université de Genève, Geneva, Switzerland and Cell Biology Laboratory, University of London, United Kingdom

Using specific rabbit antisera against AKH I and AKH II (S. Hekimi and M. O'Shea, 1989, Insect Biochem. 19:79) in immunocytochemistry, we demonstrate that the glandular lobes of the corpora cardiac (CC) consist of a homogeneous cellular population with respect to AKH biosynthesis. Another antiserum raised against a non-amidated AKH I analog allowed identification of AKH precursors, that have been sequenced (S. Hekimi et al., 1989, Neuron 2:1363; S. Hekimi and M. O'Shea, 1989, J. Neurosci. 9:996). When processed, they release active hormone and dimeric fragments formed by a single disulfide bond between 2 identical chains, APRP1 ($\alpha\alpha$) and APRP3 ($\beta\beta$) or 2 different chains, APRP2 ($\alpha\beta$). Antisera were raised against 2 oligopeptides based on the N-terminal sequences of α and β, which recognize α and β chains as well as their dimeric forms. When tested on the brain, all the antisera, except the anti-AHK I, stain numerous somata in the pars intercerbralis and axons in nervi corporis cardici I (NCCI). Staining was also occasionally observed in lateral neurosecretory cells, tritocerebral cells and NCCII. Extensive immunoreactive processes react with the β-chain and AKH II antisera. Both stain the central body complex but their staining patterns never overlap. The β-chain antiserum also stains a large number of cells and fibers in the optic lobes. The identity of these products and their relation to those in the CC remain obscure. The use of RNA probes, designed on sequences of AKH cDNA clones (M.-F. Schulz-Aellen et al., 1989, Neuron 2:1369) in hybridization histochemistry will indicate whether the immunoreactivity in the brain is due to authentic products which emerge then from differential RNA splicing. (Supported by Swiss National Fund No.3.181.0.85.)

RESISTANCE TO INSECTICIDE: DETECTION OF A MUTATION IN THE ACETYLCHOLINESTERASE GENE FROM *Drosophila melanogaster*

D. Fournier, and J.-B. Berge

INRA, BP 2078, 06606, Antibes

We cloned acetylcholinesterase gene from a *Drosophila melanogaster* strain resistant to malathion. This strain exhibits a modified Ki towards malathion and a modified Km towards acetylcholine (Morton and Singh, 1982, Biochem. Genet. 20:179-198). Sequence comparison between this gene and a wild type gene (Hall and Spierer, 1986, EMBO J., 5:2949-2954) showed only one mutation, namely a T to A substitution resulting in the replacement of a phenylalanine by a tyrosine at position 368. This phenylalanine is conserved in the two other cholinesterases sequenced so far (Torpedo acetylcholinesterase and human butyrylcholinesterase) suggesting that this amino-acid is important in the catalytic properties of cholinesterases. This mutation is important since tyrosine differs from phenylalanine both sterically (4-OH group) and electronically (OH is a good π-electron donor). Nevertheless a functional test is necessary to prove that this change is indeed responsible for the resistance.

AN AMPHIPATHIC PEPTIDE AS A POTENTIAL NEW INSECTICIDE

D.R. Frohlich, and M.A. Wells

Department of Biochemistry, University of Arizona, Tucson, AZ 85721.

Subbarao et al. (Biochemistry, 1987, 26:2964) designed a 30-residue amphipathic peptide that folded into a α-helix at low pH, disrupted lipid bilayers and resulted in lipid vesicle leakage. Accordingly, we synthesized a 30-reside peptide, designed to interact with lipid bilayers at high pH, in the hope of disrupting *Manduca sexta* gut cells. FMOC synthesis of Y-A-A-$[K\text{-}A\text{-}L\text{-}A]_6$-L-A-A resulted in 18% yield of the D-isomer and 32% yield of the L-isomer. Circular dichroism showed that both isomers are random coils at acidic and neutral pH and form α-helices at high pH. Feeding trials failed to demonstrate toxic effect to *M. sexta* larvae nor was effect demonstrated when enhanced with trypsin inhibitors. When incubated with *Spodoptera frugiperda* cells *in vitro*, cell lysis and death occur almost immediately. Additionally, 4th instar *M. sexta* injected with 100μg peptide died within 12 hours. Studies of peptide interaction with lipid vesicles were also carried out. It is likely that the heavy mucosal lining of the gut prevents the peptide from reaching gut cells and causing leakage. (Supported by NSF grant DIR-870082 and NIH grant HL-39116).

STRUCTURE-ACTIVITY STUDIES ON M1 IN *Periplaneta americana*: THE IMPORTANCE OF TERMINI AND SIDE CHAINS

G. Gäde, and T.K. Hayes

Institut für Zoologie, Lehrstuhl für Tierphysiologie, Heinrich-Heine-Universität, D-4000 Düsseldorf 1, FRG; Laboratories for Invertebrate Neuroendocrine Research, Department of Entomology, Texas Agricultural Experiment Station, Texas A&M University, College Station, TX 77843

The neuropeptide M1 (Pea-CAH I) is one of two hyperglycemic peptides in *Periplaneta americana*. The structure of the peptide demonstrates that is a member of the AKH-RPCH family of insect neuropeptides: pGlu-Val-Asn-Phe-Ser-Pro-Asn-Trp-NH_2. We have prepared several peptides by solid phase peptide synthesis for use as structural probes to find important groups in M1 for hyperglycemic activity in *P. americana*. Dose/response relationships for each analog relative to M1 suggest how M1 interacts with target cell receptors to stimulate carbohydrate mobilization. We suggest from the information provided by these studies that Phe^4 and Trp^8 are critical residues for initial binding and recognition by receptors. $Val,^2$ $Asn,^3$ Pro^6 have strong but less critical influence on receptor binding. $Asn,^3$ Ser^5, and Pro^6 have measurable influences on transduction of the biological activity. We would like to thank the Deutsche forschungrsemeinschraft (Ga 241/ 6-3 and 6-4 to GG), the Heisenberg Fellowships (GA241/5-2 to GG), USDA Grant #86-CRCR-1-2008 (to TKH) and USPHS Grant #RO1 NS20137O (to TKHO) for support of our research.

ANALYSIS OF A MUTATION IN *Bombyx mori* THAT AFFECTS CHORION ULTRASTRUCTURE

D. Gautreau, M.G. Alexopoulou, and M.R. Goldsmith

Department of Zoology, University of Rhode Island, Kingston, RI 02881-0816

Gr^{16} is a spontaneous dominant mutation of the domestic silkmoth, *Bombyx mori*, in which the helicoidal array of fibers that comprises the bulk inner chorion is perpendicular

rather than parallel to the egg surface. The mutation maps between the two known chorion structural gene clusters on chromosome 2. The first signs of abnormality appear during early chorion formation (G.D. Mazur, personal communication), suggesting that the mutation affects a critical step in the assembly of the initial extracellular framework. To study the molecular basis of the mutation, we compared mature chorion protein composition, kinetics of protein synthesis, and the gross structure of the early chorion locus in the homozygous mutant, its corresponding wild type, and the heterozygote. Two-dimensional protein patterns of mature chorions were identical, indicating that Gr^{16} expresses all of the major chorion components at normal levels. However, two pairs of minor, early proteins showed reciprocal differences in relative labeling intensities: one pair labeled more intensely in the wild type, while the other was more prominent in the homozygous mutant. These two pairs of proteins had similar molecular weights but different isoelectric points, suggesting that they are related. Preliminary pulse-chase labeling patterns were consistent with the hypothesis that the mutation affects post-translational modification of the proteins in question. Restriction analyses of genomic DNA using early chorion genes as probes revealed no difference between mutant and wild type, indicating that the mutant lacks major chromosome defects. We will present models for the molecular level at which the mutation may act, in light of these observations. (Supported by NSF grants INT 84-15135, PCM8316193, and by the Carolyn and Kenneth D. Brody Foundation.)

MECHANISM OF CELL LYSIS BY THE 24 kDa TOXIN OF *Bacillus thuringiensis israelensis*: CHARACTERIZATION OF THE CELL BINDING DOMAIN

S.S. Gill, E. Chow, and L. Shi

Department of Entomology, University of California, Riverside, CA 92521

The parasporal protein crystal produced by *B. t. israelensis* consist of at least four principal toxins, of which the 28 kDa protein is a major component, and this toxin is proteolytically cleaved to a 24 kDa polypeptide. Monoclonal antibodies were raised against both the denatured and native forms of this 24 kDa toxin. Twelve different monoclonal antibodies were produced and four of these antibodies were able to totally inhibit the cytolytic activity of the toxin. Preliminary studies showed that this decrease in cytolytic activity was due to a decrease in toxin binding to cells. Thus attempts were made to localize the cell binding domain(s) on the toxin. The 24 kDa toxin was cleaved with various proteases and gaseous cyanogen bromide and fragments separated and analyzed using immunoblot. The fragments were then sequenced and the binding domain localized to a 6 kDa fragment of the toxin. The 6 kDa peptide is within a major hydrophobic domain of the toxin, and these results indicated the significance of this domain in cell binding and cell lysis. Further independent studies using chemical modification also showed the significance of this domain. (Supported by NIEHS grant ES03298 and the University of California Mosquito Control Program Grant No. 89-07.)

MOLECULAR GENETICS OF CELL ADHESION AND CELL RECOGNITION DURING *Drosophila* DEVELOPMENT

C.S. Goodman

Howard Hughes Medical Institute, Division of Genetics, Department of Molecular and Cellular Biology, University of California, Berkeley, 94720

We are interested in using classical genetic and molecular genetic analysis to study the function of cell adhesion and cell recognition molecules during development, with particular emphasis on their role in the developing nervous system. We have been using two approaches to uncover these molecules in the fruitfly *Drosophila*. First, we have been searching for the

Drosophila homologues of well know vertebrate adhesion molecules. We cloned the genes which encode the three subunits of *Drosophila* laminin, a substrate adhesion molecule shown to be a potent promoter of neurite outgrowth in vertebrate systems. We have isolated a lethal mutation in the gene encoding the A subunit of laminin (*lama*). We also used the PCR method to clone two genes that encode *Drosophila* cadherins maps to the *fat* gene (in collaboration with Bryant's lab at U.C. Irvine), and a genetic analysis of both is presently underway.

Second, beginning with an immunological approach, we identified and subsequently cloned the genes encoding four different surface glycoproteins, which we call fasciclin I, fasciclin II, fasciclin III, and neuroglian. These glycoproteins are dynamically expressed on different overlapping subsets of axon fascicles and glia during embryonic development. Two of these molecules (fasciclin II and neuroglian) are part of the immunogloubin superfamily and are highly related to a series of vertebrate neural cell adhesion molecules (fasciclin II is highly related to N-CAM and Neuroglian to L1). The other two proteins fasciclin and III) uncovered by our immunological screen are novel structures. Using transfection, aggregation, and biochemical assays, we have shown that fasciclin III and fasciclin I are both homophilic adhesion molecules which appear to define new classes of adhesion molecules. We have identified small deletions and/or point mutations in the neuroglian, fasciclin I and fasciclin III genes and are using genetic analysis to study the function of these molecules in the developing organism.

CUTICULAR HYDROCARBON ACCUMULATION AND BIOSYNTHESIS OF THE SOUTHERN ARMYWORM, *Spodoptera eridania*

L. Guo and G.J. Blomquist

Department of Biochemistry, University of Nevada, Reno, NV 8944-0014

The accumulation biosynthesis of cuticular and internal hydrocarbons in the southern armyworm, *Spodoptera eridania*, were examined at closely time intervals during larval and pupal and dimethylalkanes. The amount of cuticular hydrocarbon stayed relatively constant during each stadium while the amount of internal hydrocarbon increased dramatically during the first half hydrocarbon on the shed cast skin with each molt. The accumulation of internal hydrocarbon was mirrored by large increases in the rate of incorporation of labeled acetate into the hydrocarbon fraction. The incorporation of radiolabeled valine, isoleucine, methionine and succinate into hydrocarbon indicated that these amino acids and branched alkanes. Radio-HPLC was used to examine intermediates in the conversion of valine and succinate to propionate and methylmalonate.

ABERRANT TRANSCRIPTION OF THE *Autographa californica* NUCLEAR POLYHEDROSIS VIRUS (ACMNPV) GENOME IN TWO INSECT CELL LINES SEMIPERMISSIVE FOR ACMNPV REPLICATION

D. Guzo and E.M. Dougherty

USDA, ARS, Insect Pathology Laboratory, Beltsville, MD 20705

The gypsy moth cell lines IPLB-Ld652Y and IPLB-LdFB have been shown to be semipermissive for replication of the AcMNPV. Viral protein synthesis, as determined by ^{35}S methionine pulse labeling and SDS-PAGE, appears to be aborted in the α phase. In both cell lines, however, all viral and host cell protein synthesis is inhibited after 24 hrs post infection. Quantitative and qualitative analyses of AcMNPV specific transcription products indicates that while the overall levels of viral specific m-RNAs are equivalent in infected semipermissive and permissive cell lines, some viral transcripts in infected IPLB-Ld652Y and LdFB cells are of

considerably higher molecular weight than homologous transcripts in cell lines permissive for virus replication. The aberrant viral transcripts, which are not translated in infected cells, are believed to result from a breakdown in the normal mechanisms regulating AcMNPV specific transcription in the gypsy moth cell lines. The subsequent infection mediated total inhibition of all cellular and viral protein synthesis appears to occur via a second, as yet undermined, mechanism involving a total shutoff of normal host cell translation of m-RNAs.

BIOCHEMICAL ALTERATIONS IN THE PERITROPHIC MEMBRANE FOR *Trichoplusia ni*

R.R. Granados, Y. Hashimoto, B. Corsaro, and L.G. Gallo

Boyce Thompson Institute for Plant Research, Cornell University, Ithaca, NY 14853

Major structural changes in the peritrophic membrane (PM) occur upon feeding certain baculoviruses to *Trichoplusia ni* larvae. Analysis of temporal changes in protein composition of the PM, following inoculation with nuclear polyhedrosis viruses (NPV), showed that concurrent with morphological and structural changes, one of the glycoproteins (gp68) disappeared from the PM. In contrast to the NPVs two different granulosis viruses (GV) degraded PM-gps 253, 194, and 123 to several lower molecular weight gps. A newly discovered virulence gene product known as a viral enhancing factor (VEF) and present in the occlusion bodies of several granulosis viruses in a unique viral-encoded protein which enhances virus infection in larvae. Studies on the mode of action of the VEF isolated from *T. ni* GV (TnGV) showed that the VEF caused rapid degradation of the PM which lines the midgut lumen and possibly assisted in the passage of virions across this presumptive barrier. We have cloned and sequenced the VEF gene from TnGV and showed sequence homology of the VEF virulence gene among different GVs. Our findings showed that GV occlusion bodies may serve as vehicles for the delivery of biologically active proteins which are important in the early events of virus infection in insect hosts.

ISOLATION AND CHARACTERIZATION OF A HOST HEMOLYMPH PROTEIN REQUIRED FOR DEVELOPMENT OF THE EGGS OF THE ENDOPARASITE *Microplitis croceipes*

P. Greany,[1] W. Clark,[1] S. Ferkovich,[1] J. Law,[2] and R. Ryan[3]

[1] Insect Attractants, Behavior & Basic Biology Research Laboratory, USDA ARS, P.O. Box 14565, Gainesville, FL 32604
[2] Department of Biochemistry, University of Arizona, Tucson, AZ 85721
[3] Department of Biochemistry, University of Alberta, Edmonton

Newly-laid eggs of the braconid *Microplitis croceipes*, a solitary larval endoparasite of *Heliothis* spp. larvae, were found to require the presence of a host hemolymph protein to initiate embryogenesis *in vitro*. A two-step purification system was developed to acquire the egg development stimulating protein (EDSP) from *H. zea* hemolymph using gel permeation chromatography followed by DEAE high performance liquid chromatography. EDSP has a native molecular weight of ca. 230 kDa, with subunits appearing between 15.5 and 43 kDa on SDS PAGE gels. Medium preconditioned by culturing host fat body yielded significant EDSP activity, suggesting that fat body is the biosynthetic source. EDSP activity also was present in blood of a number of non-permissive hosts, including *Manduca sexta*, *Trichoplusia ni*, *Anticarsia gemmatalis,* and *Spodoptera exigua*.

EXPRESSION DENSONUCLEOSIS VIRUS *Gm*DNV IN *Galleria mellonella* LARVAE

O. Gross,[1] D. Weinberg,[1] P. Tijssen,[2] and J. Tal[1]

[1] Biology Department, Ben-Gurion University of the Negev. Beer-Sheva 84105, Israel
[2] Institut Armand-Frappier, Universite du Quebec, Laval-des-Rapides, Quebec, Canada H7N 4Z32

The densoviruses (DNV), genus within the parvovirus family, are small viruses (ca. 20nm in diameter) which are pathogenic to a variety of invertebrates. Their genome is a single-stranded DNA molecule of 5 to 6 kilobases (kb) with long inverted terminal repeats (0.44 kb). Their limited coding capacity makes them completely dependent on the host, and thus serve as sensitive probes for its cellular activities. Since densoviruses cannot be propagated in established cell cultures, the study of their replication and gene expression is restricted to *in vivo* systems.

The size analysis of a densovirus RNA is reported here for the first time. The RNA of densonucleosis virus type 1 (*Gm*DNV), isolated from *Gm*DNV-infected *Galleria mellonella* larvae, was 2.4 3.5, and 4.0 and 5.0 kb. This RNA was translated in a rabbit reticulocyte lysate system, and the translation products were co-electrophoresed with proteins extracted from CsCl-purified virus. The most abundant *in vitro* translation product co-migrated with the smallest, 46 kDa viral polypeptide, indicating that the latter is a primary translation product. Furthermore, the most abundant RNA species, the 1.8 kb transcript (about 50% of the total RNA), is only slightly larger than the template required for a polypeptide of this size. The 46 kDa polypeptide is not, however, the most abundant protein in purified virions, suggesting translational or post-translational control. These results suggest that there are striking similarities in gene expression of densoviruses and mammalian parvoviruses.

LOCALIZED GENE EXPRESSION IN THE SALIVARY GLANDS OF THE MOSQUITO, *Aedes aegypti*

G.L. Grossman [2] and A.A. James[1]

[1] Department of Molecular Biology and Biochemistry, University of California, Irvine, 92717
[2] Department of Tropical Public Health, Harvard School of Public Health, Boston, MA 02115

We are developing methods for expressing genes in mosquitoes using endogenous promoter DNA sequences. As part of our characterization of suitable promoters, we are looking at the tissue-specificity of several genes expressed in the salivary glands of adult *Aedes aegypti*. Hybridizations *in situ* with antisense RNA probes have been used to identify the cells in the salivary glands that express the genes. We have found that morphologically distinct lobes of the salivary glands express a different subset of the genes examined, and that this expression is correlated with the sex-specificity of the genes. For example, Maltase-like I, a gene expressed in both males and females, is expressed in all lobes of the male salivary glands, but only the proximal lateral lobes of the female salivary gland. However, the gene D7, expressed preferentially in females, is expressed in a complementary pattern. All regions of the female salivary gland express D7 except the proximal lateral lobes. The relationship between sex-specific and lobe-specific expression is correlated with cellular differences observed microscopically by earlier workers. Differences in expression are presumably related to the ability of the female to bloodfeed.

A SINGLE RADIAL IMMUNODIFFUSION TECHNIQUE USED TO ANALYZE THE DIGESTIVE PHYSIOLOGY OF INSECTS REARED ON AN ARTIFICIAL DIET

J.R. Hagler and A.C. Cohen

Honey Bee Biology and Biological Control of Insects, USDA-ARS, 2000 East Allen Road, Tucson, AZ 85719

Rabbit antigen (IgG) was incorporated into a predaceous heteropteran's artificial diet. The predators (*Geocoris punctipes)* were allowed to feed one hour then held at various times and temperatures. Guts of individual *G. punctipes* were then macerated in phosphate buffer and 10-μl aliquot was pipetted on a agar gel containing rabbit antibody (anti-IgG). Antigen was detectable in the gut 48 hours after ingestion at 27 °C. Digestive rates increased with increasing temperatures. Applications of this single radial immunodiffusion technique studying the digestive physiology of insects reared on artificial diet are discussed.

EFFECTS OF PARASITISM BY *Cotesia congregata* ON THE HEMOCYTE RESPONSE OF THE TOBACCO HORNWORM, *Manduca sexta*

C.A. Hallberg and P.E. Dunn

Department of Entomology, Purdue University, West Lafayette, IN

In order for the eggs of a parasitoid to develop within the hemocoel of its host, the defensive responses of the host must be survived or evaded. The defense response of insects to large objects such as parasitiod eggs is encapsulation by hemocytes. Secretions of the female wasp's reproductive tract, venom and polydnaviruses, have been shown to be involved in parasitoid nullification of host hemocyte encapsulation.

Parasitism of *Manduca sexta* larvae by the braconid parasitoid *Cotesia congregata* results in a rapid increase in susceptibility of host larvae to bacterial challenges easily survived by naive, nonparasitized *M. sexta* larvae (D. Ross and P. Dunn, Devel. Comp. Immunol., 1989, 13:205-216). To determine which reproductive tract secretions injected by the female wasp during oviposition were necessary for destroying the hemocyte-mediated antibacterial responses, fluids from various portions of the female wasp reproductive tract were injected into *M. sexta* larvae, which were subsequently challenged with *Pseudomonas aeruginosa* strain 9027. Injection of either venom or calyx fluid alone was not sufficient to increase host susceptibility to bacteria. Injection of the two components together, however, produced a rapid increase in host susceptibility, paralleling natural parasitism. This suggest that both venom and calyx fluid, which contains large amounts of a polydnavirus in *C. congregata*, are necessary factors in disabling host hemocytes.

The severe effect of parasitism on host hemocyte function is accompanied by changes in hemocyte morphology. Changes in the structure of hemocytes upon parasitization were examined using fluorescent probes for actin and tubulin cytoskeletal elements.

PROTEASE INHIBITOR ACTIVITY, IMMUNOREACTIVITY, AND GENE EXPRESSION IN THE DEVELOPING ANTENNAE AND BRAIN OF *Manduca sexta*

E.H. Hanneman, M.R. Kanost, H.K. Lehman, and J.G. Hildebrand

Arizona Research Laboratories Division of Neurobiology and Department of Biochemistry, University of Arizona, Tucson, AZ 85721

Proteases and their inhibitors are expressed at critical stages in the development of individual neurons and in the overall pattern of development. Inhibition of proteases released

from neuronal growth cones decreases cell migration and sprouting, and glial cells can release protease inhibitors. Proteases also have global roles in pattern formation, such as the *snake* and *easter* mutations that affect the dorsal-ventral axis in *Drosophila*.

The brain of *Manduca sexta* undergoes extensive remodeling during pupal development, a period when larval networks are degenerating and new adult structures form. We used a simple assay to show that stage-18 pupal brain contains protease inhibitor activity against trypsin and chymotrypsin. Northern analysis at low stringency using a serine protease inhibitor (serpin) cDNA isolated from *Manduca* fat body shows that pupal brain and antennae contain up to 4 transcripts that hybridize to the 1.4 kb serpin cDNA. Antibodies generated against three different serpins isolated from hemolymph demonstrate three different patterns of expression during brain development. These antibodies have been used to isolate several clones from a stage-18 brain cDNA expression library.

The cloning of these transcripts and study of their localization will help to determine how proteases and their inhibitors affect the restructuring of the *Manduca* nervous system during development. (Supported by grants from the NIH, NSF, and ACS).

EXPRESSION OF JUVENILE HORMONE ESTERASE IN *Bombyx mori* LARVAE AND CELL CULTURE USING A BACULOVIRUS VECTOR

T.N. Hanzlik, B.C. Hammock, and S. Maeda

Department of Entomology, University of California, Davis, CA 95616

A recombinant transfer vector for *Bombyx mori* nuclear polyhedrosis virus (BmNVP) was constructed that contained a full cDNA coding for juvenile hormone esterase (JHE) from *Heliothis virescens*. BmN cells were transfected with the recombinant vector and DNA of wild type BmNPV and a recombinant virus was isolated. The recombinant virus expressed JHE activity in both infected BmN cell culture and *B. mori* larvae from several instars. Transcription of the JHE mRNA produced by cell culture and larvae was analyzed by Northern Blotting. The JHE protein produced by cell culture and larvae was compared to purified JHE from *H. virescens* with immuno-blotting and kinetic analysis. The biological effect of JHE in four of the five larval instars was examined by weight gain and observation of molting patterns.

SEQUENCE DIFFERENCES AMONG THREE cDNA CLONES CODING FOR JUVENILE HORMONE ESTERASE FROM *Heliothis virescens*

T.N. Hanzlik, S. Maeda, L. Harshman, and B.D. Hammock

Department of Entomology, University of California, Davis, CA 95616

Three clones coding for Juvenile Hormone Esterase from *Heliothis virescens* were isolated from a size selected, nonamplified cDNA expression library made in a derivative of λgt11 λZAP). The cDNA was made from mRNA isolated from last instar larvae that had been previously treated with a juvenoid. The cDNA clones were isolated after screening the library with a monospecific antibody and a mixture of oligonucleotides complementary to the mRNA transcript's 5′ region coding for the N-terminus of the protein. The three cDNA clones are highly similar yet display distinct differences among them. All are approximately 3,000 base pairs in length which matches the length of the single mRNA transcript detected on a Northern Blot. The clones have identical restriction patterns with certain restriction endonucleases yet differ with other enzymes. The base difference among the clones, some of which lead to amino acid changes, are on the order of one base difference per 100 bases when analyzed by sequencing. The effect and genesis of the differences are discussed.

CONTROL OF EXPRESSION OF *Heliothis virescens* JUVENILE HORMONE ESTERASE

L. Harshman,[1] B. Bonning,[2] B.D. Hammock,[1] T. Hanzlik,[1] R. Posse,[2] and V. Wroblewski[1]

[1] Department of Entomology, University of California, Davis, CA
[2] NERC Institute of Virology and Environmental Microbiology, Oxford, U.K.

Juvenile hormone esterase (JHE) from *Heliothis virescens* has been cloned and sequenced. An N-terminal fragment from the cloned cDNA was used to monitor the abundance of JHE message in concert with western blot analysis. This work indicates that JHE development regulation and control of induction may occur at the level of transcription. In order to begin the process of studying gene regulation, a *H. virescens* genomic library has been constructed and screened with 5´ and 3´ probes from the JHE cDNA.

Baculovirus expression of JHE has been accomplished in insect cell lines and in larvae. Specifically, a *Bgl*II linker was added to the 5´ end of the *H. virescens* cDNA. A majority of the non-coding 3´ region was removed and a second *Bgl*II linker added just downstream from the natural translation stop codon. Co-transfection of *Autographa californica* nuclear polyhedrosis virus with a transfer vector containing the JHE cDNA produced recombinate viruses which gave high levels of expressed JHE from *Spodoptera frugivera* (SF) cells. JHE activity appeared primarily in tissue culture medium suggesting that the leader sequence of the JHE cDNA is an efficient export signal in SF cells. *In vivo* expression of JHE was monitored after feeding recombinant polyhedron-minus viruses to *Trichoplusia ni.*

FATTY ACID BINDING PROTEIN FROM LOCUST FLIGHT MUSCLE CYTOSOL

N.H. Haunerland and J.M. Chisholm

Department of Biological Sciences, Simon Fraser University, Burnaby, B.C. V5A 1S6, Canada

The desert locust *Schistocera gregaria* continues to be a major threat to developing nations in Africa and Southeast Asia. Novel strategies focus on interfering with locust swarming and migration. Since extended flight during migration is fueled almost exclusively by lipid, our studies concentrate on aspects of lipid metabolism in the flight muscle.

During sustained flight, diacylglycerol is delivered to the muscle tissue after mobilization in the fat body and transport by lipophorin through the hemolymph. Following hydrolysis of the diglyceride, free fatty acid enters the muscle cell. However, fatty acids are only poorly soluble in the hydrophilic cytosol. Therefore, a special transport mechanism seems to be necessary for their efficient translocation to the mitochondria, where β-oxidation occurs.

Our studies with flight muscle cytosol revealed the existence of a low molecular weight fatty acid binding protein (FABP) which might be responsible for this transport. The protein was purified by gel permeation chromatography and isoelectric focusing. Its molecular properties are reminiscent of mammalian M-FABP, which has been shown to be essential for β-oxidation in heart muscle.

Our discovery, showing for the first time a structurally similar FABP in invertebrate tissue, highlights the importance of fatty acid binding proteins in lipid metabolism.

MOLECULAR PHARMACOLOGY AND METABOLISM OF INSECT HYPERTREHALOSEMIC HORMONE

T.K. Hayes, A. Strey, M. Ford, and L.L. Keeley

Laboratories for Invertebrate Neuroendocrine Research, Department of Entomology, Texas Agricultural Experiment Station, Texas A & M University, College Station, TX 77843

Blaberus discoidalis hypertrehalosemic hormone (HTH pGlu-Val-Asn-Phe-Ser-Pro-Gly-Trp-Gly-Thr-NH_2) is a member of the AKH/hyperglycemic hormone family. HTH elevates hemolymph trehalose concentrations by 3.5 fold in *Blaberus*. Several series of synthetic structural probes have been prepared to help identify specific side chain, termini and conformational structural features that are important for the transmission of biological information. Dose/response relationships for each analog relative to HTH suggest how HTH interacts with target cell receptors to stimulate carbohydrate mobilization. $pGlu,^1$ Phe^4 and Trp^8 appear to be the most important residues for receptor recognition and binding. D-amino acid placement in active analogs, amino acid frequency data and analog potency correlations suggest the presences of a β-turn around 5-8. Aromaticity is required at positions 4 and 8. Further, a bicyclic aromatic ring is required at position 8. Ser^5 and the neutral C-terminal amide are important for transduction of the biological response. (Supported by USDA #86CRCR-1-2008 and USPHS #RO1 NS20137.)

A NOVEL FEMALE-SPECIFIC PROTEIN PRODUCED BY THE VITELLOGENIC FAT BODY AND ACCUMULATED IN OVARIES IN THE MOSQUITO *Aedes aegypti*

A.R. Hays and A. Raikhel

Department of Entomology, Michigan State University, East Lansing, MI, 48824

In addition to vitellogenin, the fat body of vitellogenic mosquito females was found to synthesize and secrete another protein which accumulated in ovaries. On SDS-PAGE gels this protein has ca. Mr=53,000 (53KP). Rabbit polyclonal antibodies were produced using 53 kDa peptide purified by preparative SDS-PAGE from vitellogenic ovaries as the antigen. Radioimmunoassay developed using these antibodies showed that the synthesis of 53KP by the female fat body begins before 4 hours after the initiation of vitellogenesis by blood feeding and has ceased to be detectable after 48 hours. Peak synthesis is near 24 hours after blood is taken. This protein is glycosylated as demonstrated by $[^3H]$mannose incorporation and experiments with tunicamycin. The experiments with tunicamycin further demonstrated that the glycosylation accounts for ca. 1.2 kDa of the 53 kDa. The protein is not detectably phosphorylated or sulfated. *In vitro* experiments conducted on fat bodies of previtellogenic females demonstrated that the synthesis and secretion of 53KP can be stimulated by 10^{-6} M 20-hydroxyecdysone. (Supported by NIH grants AI-24716 and HD-22958.)

GENETIC LINKAGE ANALYSIS OF A LOCUS CONTROLLING ACETYLCHOLINESTERASE SENSITIVITY TO ORGANOPHOSPHATE INSECTICIDES IN THE TOBACCO BUDWORM, *Heliothis virescens*

D.G. Heckel, P.K. Bryson, and T.M. Brown

Departments of Biological Sciences and Entomology, Clemson University, Clemson, South Carolina 29634

Organophosphate insecticides (OPs) are toxic because they inhibit the enzyme acetylcholinesterase (AChE), and one mechanism of OP-resistance is possession of a form of AChE that is less sensitive to inhibition. An OP-insensitive AChE is present in field and laboratory strains of *Heliothis virescens*, and its expression appears to be controlled by a single locus with two alleles (Brown and Bryson, these proceedings). Our working hypothesis is that this is a structural locus encoding AChE. We attempted to map it relative to other marker isozyme loci segregating in the PTJ laboratory strain, in which half of the 31 chromosomes of *H. virescens* are marked. The AChE locus was found to be located on linkage group 2, which also contains the loci for one of the isozymes of isocitrate dehydrogenase and one of the isozymes of glutamate-oxaloacetate transaminase (both of which are encoded by two loci in *H. virescens*). The AChE locus is located approximately 11 centimorgans away from the former. It segregates independently from all other isozyme markers in the PTJ strain. This is the first linkage group assignment for a gene conferring insecticide resistance in major lepidopteran crop pest.

cDNA AND GENOMIC CLONES FOR THE LARGE YOLK PROTEIN OF THE BOLL WEEVIL *Anthonomus grandis*.

L.J. Heilmann,[1] P.M. Trewitt,[2] S.S. Degrugillier,[1] and A.K. Kumaran[2]

[1] USDA-ARS Biosciences Research Laboratory, Fargo, ND
[2] Department of Biology, Marquette University, Milwaukee, WI

The yolk proteins, or vitellogenins, are a family of abundant proteins synthesized in the female fat body and stored in the developing egg. Yolk proteins serve as a model system for studying the sex-specific control of gene expression, and their wide phylogenetic distribution also makes them ideal for evolutionary comparisons. Eggs of the cotton boll weevil, *Anthonomus grandis*, contain two major yolk proteins 47 kDa and 160 kDa in size. We prepared polyclonal antibodies to the large yolk protein and used them to screen an adult female cDNA library cloned in λgtll. Several clones complementary to a 6.5 kilobase female-specific message were isolated. We also screened a female λgt10 library with both adult male and female single strand cDNAs, and isolated a female-specific 1200 bp clone also complementary to the 6.5 kb female-specific message. Genomic Southern blots show that this sequence is encoded by a single copy gene. The two cDNA clones were used to screen an *A. grandis* genomic EMBL3 library. Several overlapping clones covering the yolk protein locus have been isolated and the relevant portions mapped and sequenced. No homology is observed with other published yolk protein sequences.

ISOLATION AND STRUCTURE ELUCIDATION OF NOVEL 5-kDa PEPTIDES FROM NEUROHAEMAL LOBES OF THE CARDIACA CORPORA OF *Locusta migratoria*

H. Hietter,[1] A. Van Dorsselaer,[1] B. Green,[2] L. Denoroy,[3] J. Hoffmann,[4] and B. Luu[1]

[1] Laboratoire de Chimie Organique des Substances naturelles, UA 31 du C.N.R.S., 5 rue Blaise Pascal 67000 Strasbourg, France
[2] VG Analytical Ltd., Manchester, England
[3] Service central d'analyse du C.N.R.S., Vernaison, France
[4] Laboratoire de Biologie Générale, UA 672 du C.N.R.S. "Endocrinologie et Immunologie des Insectes", 12 rue e l'Université 67000 Strasbourg, France

Two predominant peptides have been isolated from neurohaemal lobes of corpora cardiaca of 8,000 adults of *Locusta migratoria*. Both peptides have been unambiguously characterized by automated peptide microsequencing and liquid secondary-ion mass spectrometry as 50-residue peptide (5 K-peptide) and a 48-residue isologue (5 K′-peptide). Computer search of sequence data banks did not reveal any significant homology with other identified proteins. The 5 K-peptides are remarkably rich in alanine residues (25%) and namely contain a stretch of 5 consecutive alanines. This structure suggests that these molecules could correspond to spacer peptides. This assumption is corroborated in the accompanying presentation (Lagueux, Lwoff, Meister, Goltzené and Hoffmann) on the molecular cloning of the precursor protein which attributes to the 5 K-peptides a positional analogy to the C-peptides of insulins.

REGULATION OF DOPA DECARBOXYLASE GENE EXPRESSION IN THE LARVAL EPIDERMIS OF THE TOBACCO HORNWORM BY 20-HYDROXYECDYSONE AND JUVENILE HORMONE

K. Hiruma, M.S. Carter, and L.M. Riddiford

Department of Zoology, University of Washington, Seattle, WA 98195

Dopa decarboxylase (DDC) is important for cuticular melanization as well as sclerotization in *Manduca sexta* larvae. Using the *Drosophila* DDC gene, we have isolated the *Manduca* DDC gene and are sequencing it and its cDNA. This gene selects a 3.1 kb mRNA from the epidermis which encodes a 49 kDa polypeptide precipitable by the *Drosophila* DDC antibody. DDC mRNA appears in the epidermis 12 hr after head capsule slippage (HCS) during the molt to the 5th instar and attains maximal levels 7 hr later, then declines to low level by the time of ecdysis. This increase in DDC mRNA could be prevented by infusion of 1μg 20-hydroxyecdysone/hr for 18 hr beginning 2 hr after HCS or by culturing this epidermis with 1 μg/ml 20-hydroxyecdysone for 24 hr. Preliminary experiments with cycloheximide suggest that protein synthesis may be necessary for the suppressive action of 20-hydroxyecdysone. Culture of day two, 4th instar larval epidermis showed that an initial exposure to more than 0.3 μg/ml 20-hydroxyecdysone for 12-18 hr followed by 24 hr in hormone-free medium was necessary for this later increase in DDC expression. Maximal induction occurred with 2 μg/ml 20-hydroxyecdysone. The maximal level of DDC mRNA in the epidermis of a melanizing allatectomized larvae was 2-fold higher than that in intact larvae could be depressed to normal level by application of 0.1 μg juvenile hormone I within four hr of HCS. Thus, ecdysteroid during a larval molt is first necessary to determine the later expression of DDC and at this time the presence or absence of juvenile hormone can influence the level of expression. The subsequent decline of ecdysteroid is required for this expression to occur. (Supported by NIH AI12459 and NSF PCM85-10875.)

THE DEGREE OF GENETIC VARIABILITY IN PARTHENOGENETIC HONEY BEES

G.D. Hoffman,[1] G.A. Hoelzer,[2] M. Spivak,[3] and M. Kidwell[2]

[1] Carl Hayden Bee Research Center, USDA-ARS, Tucson, AZ
[2] Department of Ecology and Evolutionary Biology, University of Arizona, Tucson, AZ
[3] Center for Insect Science, University of Arizona, Tucson, AZ

A strain of honey bee (*Apis mellifera* L.) has the ability to requeen colonies using the eggs of laying workers. The strain will hereafter be referred to as Lusby bees (LUS). This trait (thelytokous parthenogenisis) is rare in European honey bees but commonly occurs in the South African Cape honey bee (*A. mellifera capensis*). The degree of genetic variability among individuals from LUS and non-thelytokous honey bee lines was determined using DNA fingerprinting.

Genomic DNA from individual worker, queen, and drone honey bees was extracted and then cut using restriction enzymes. The resulting DNA fragments were separated using gel electrophoresis, and then transferred to a nylon membrane. The DNA on the membrane was probed for fragments carrying particular variable number tamden repeats (VNTR's) using a non-radioactive "Genius Kit."

DNA from individual worker bees from two different unrelated colonies was cut with *Hin*dIII and probed with M-13. There were no unique bands among any individuals within a colony or between the two colonies. Next, DNA from workers, their queen mother, and drone brothers was cut with *Hae*III and probed with an oligonucleotide. $[(CAC)_5]$. There were no bands in the workers that did not also appear in the queen mother or drone brothers. Similar results were obtained when the DNA was cut with *Eco*R1 and probed with M-13. Results indicate that the VNTR's from these probes are not variable in honey bees and that other probes are needed to find variability among individuals.

ECDYSTEROID PROFILE IN DEVELOPING OVARIES AND EGGS OF *Spodoptera littoralis*

N. Hoggard, and H.H. Rees

Department of Biochemistry, University of Liverpool, P.O. Box 147, Liverpool, L69 3BX, U.K.

Ecdysteroids not only occur in immature stages of insects, but also in the ovaries of maturing adult females and in eggs. The major ecdysteroids occurring during oogenesis and embryogenesis in the cotton leafworm, *Spodoptera littoralis*, have been analyzed. Initially, the metabolism of injected $[^3H]$cholesterol was examined to indicate the possible ecdysteroids occurring. Four principal metabolites were characterized by physical methods, 2-deoxyecdysone, 2-deoxyecdysone 22-phosphate and, unusually, the corresponding 5α-epimers. It is possible that the latter isomers do not occur *in vivo*, but arise by keto-enol tautomerism during isolation. Surprisingly, only small amounts of ecdysone and 20-hydroxyecdysone were identified during embryogenesis by HPLC/RIA.

In the ovaries of *Spodoptera*, the total ecdysteroid concentration remains fairly constant following the first day of ovarian development until egg laying two days later, suggesting that synthesis occurs before this. Appreciable amounts of ecdysteroids are passed into the eggs, where the titre per batch of eggs decreases early in embryogenesis, but apparently rises again at a later stage before hatching. Surprisingly, there was little apparent metabolism of the ecdysteroids during embryogenesis.

MOLECULAR ANALYSIS OF ECLOSION HORMONE IN THE TOBACCO HORNWORM, *Manduca sexta*

F.M. Horodyski, L.M. Riddiford, and J.W. Truman

Department of Zoology, NJ-15, University of Washington, Seattle, WA 98195

Eclosion hormone (EH) is a 62 amino acid neuropeptide which initiates the ecdysis behavior of insects. We isolated the genomic and cDNA clones for EH from the tobacco hornworm, *Manduca sexta*. The gene is 7.8 kb in length and is composed of 3 exons. Sequence analysis of the cDNA clone showed that the translated regions of the EH mRNA encodes an 88 amino acid precursor which consists of a 26 amino acid signal peptide and a single copy of the EH peptide. The gene is single copy in the *Manduca* genome. The EH gene is expressed as a single 0.8 kb transcript in the brain of larvae, developing adults, and diapausing pupae. The gene is not expressed in the larval nerve cord or epidermis. We showed by *in situ* hybridization that the EH mRNA was present only in the 2 pairs of ventromedial neurosecretory cells in the brain of feeding 5th instar larvae, and developing adults at days 10, 15 and 18 after pupation. Hybridization was not detected to any other cells in the brain these times. Low stringency hybridization of the *Manduca* EH gene to DNA from *Bombyx mori* and *Drosophila melanogaster* showed the presence of a single cross-hybridizing region in the genomes of both insects. (Supported by USDA 85-CRCR-1-1756 and NSF 8719611).

GENE AND GENOME STRUCTURE IN THE BLOWFLY *Lucilia cuprina*: MOLECULAR STUDIES ON EYE COLOR GENES

A.J. Howells

Department of Biochemistry, Faculty of Science, Australian National University, Canberra, Australia

The homologues of the *white* and *scarlet* genes of *D. melanogaster* have been cloned from *L. cuprina* and extensive sequence comparisons have been undertaken. Generally speaking, the coding regions of the genes have been strongly conserved between the species, the exons being about 80% homologous when the derived amino acid sequences are compared. In most cases introns interrupt the coding sequences at precisely the same points (although there appears to be one exception) but there has been little conservation of intron sequences. Two major differences in genome organization have been found between the species. Firstly, the *D. melanogaster* genome in these regions is much more CG-rich (50% G+C compared with 29% for *L. cuprina*); this applies in exons as well as introns and is accompanied by dramatic changes in the pattern of codon usage. The second difference involves the prevalence of interspersed repeated sequence DNA which is absent from the *D. melanogaster* genes but is prevalent in and around those from *L. cuprina*.

GENES OF THE HUMORAL IMMUNE RESPONSE IN *Drosophila*

D. Hultmark, D. Kimbrell, P. Kylsten, and C. Samakovlis

Department of Microbiology, University of Stockholm, S-106 91
Stockholm, Sweden

For many insects the major humoral defense against bacteria is provided by an elaborate set of antibacterial peptides which are synthesized after an infection. So far, five different classes of antibacterial peptides have been found in insects; cecropins, attacins, diptericins, defensins and lysozyme. We isolated genomic clones for the cecropin and lysozyme genes in *Drosophila*, using heterologous probes at reduced stringency. For cecropin, we found several overlapping clones from a single locus. This locus is organized as an extremely compact cluster of three cecropin genes and two pseudogenes, as well as a possibly cecropin-related gene, all within 4 kb of DNA sequence. The *Drosophila* cecropins are closely related to previously known dipteran cecropins. Transcription of the genes is dramatically induced in fat body cells of larvae and adults when the immune response is triggered by the injection of bacteria. The lysozyme locus was similarly organized as a single cluster, containing at least five genes. The expression of two genes was followed, one being expressed in the salivary gland, the other in distinct sections of the digestive tract. None of them was induced by bacteria. Using these and other cloned genes from *Drosophila* we plan to study the molecular basis of the immune response in insects.

IMMUNOCYTOCHEMICAL COMPARISON OF INSECT NEUROPEPTIDES AND REPRODUCTION RELATED PEPTIDES OF THE SNAIL *Lymnea stagnalis.*

R. Huybrechts, W. Theunis, and A. De Loof

Zoological Institute of the Katholieke Universiteit Leuven, Naamsestraat
59, B-3000 Leuven, Belgium

By use of an immunocytochemical comparison with reproduction related peptides of the pond snail *Lymnaea stagnalis* we hope to find a new start for the identification of unknown peptides in insects. Antisera against the ovulation hormone (CDCH) as well as against α and β CDCP of the snail were chosen since in this species those 3 peptides are encoded for by a single gene and are co-expressed in the caudodorsal cells. Provided that a comparable precursor would be present and a similar processing occurs in insects these antisera have to result in co-localisation.

In our study 4 insect species representing Diptera, Coleoptera and Orthoptera, are involved. In *Sarcophaga* and *Leptinotarsa* all three antisera react positively but only in the former one co-localisation with all three antisera is observed. In *Periplaneta* and *Locusta* the anti-β CDCP antiserum is negative but both other antisera give clear co-localisation in several cells of the central nervous system.

In vitro EXPRESSION OF PROUROKINASE BY A RECOMBINANT *Bombyx mori* BACULOVIRUS

R. Huybrechts

Zoological Institute of the Katholieke Universiteit Leuven, Naamsestraat
59, B-3000 Leuven

Single chain urokinase type plasminogen activator (Scu-PA) is a component of the fibrinolytic system and has significance in thrombolytic therapy. Scu-PA can now be obtained

in relatively high amounts by recombinant DNA technology. Bacterial expression results in non glycosylated bioactivated product. Expression in a mammalian system (insertion upstream a SV 40 promotor) gives a glycosylated bioactive product. However, bioactivity decreases upon prolonged culture and secretion *in vitro*.

In order to explore an alternative expression system and to evaluate the relative importance of glycosylation, we cloned Scu-PA cDNA in a -8 deletion mutant of *Bombyx mori* nuclear polyhedrosis virus (Iatrou, K., and Meidinger, R.G., 1989, Gene, 75:59-71). After cotransfection with wildtype viral DNA and following *in vivo* recombination, we successfully cloned recombinant virus by occlusion minus selection in a plaque assay.

This recombinant virus only gives expression levels up to 20 ng/ml of supernatants. To explain this low expression we are now checking the predicted polyhedrin promotor control and the intactness of this promotor.

SOLUBILIZATION, IDENTIFICATION AND LOCALIZATION OF OVARIAN BINDING SITES FOR VITELLOGENIN IN THE COCKROACH *Nauphoeta cinerea*

L.S. Indrasith,[2] H. Kindle,[1] and B. Lanzrein[1]

[1] Division of Animal Physiology, University of Berne, Erlachstr. 9a, CH-3012, Berne, Switzerland
[2] Present address: Kubota Institute for Biotechnology, Kubota Ltd., Amagasaki, Hyogo, Japan

Ovarian binding sites for vitellogenin were solubilized and identified by SDS-PAGE combined with Western blotting, followed by incubation with ^{14}C-vitellogenin and autoradiography. We tested SDS, Chaps, octyl-ß-D-glucoside and sodium deoxycholate and found specific vitellogenin binding sites after incubation with SDS and sodium deoxycholate only. One binding site had an apparent molecular weight of about 30,000 and the three others of 14-20,000. Analysis of ootheca and yellow bodies(= follicle cell/basal lamina complex which remains in the oocyte chamber) in newly pregnant females and separate analysis of oocyte plasma membrane fraction and follicle cell/basal lamina fraction in vitellogenic follicles revealed that the 30,000 binding site is located in the oocyte plasma membrane while three other binding sites are located in the follicle cell/basal lamina complex. In a filter assay only sodium deoxycholate-solubilized extracts showed binding activity after dilution of the solubilizer below its critical micellar concentration. Binding of vitellogenin to all 4 binding sites was sensitive to suramin.

CHARACTERIZATION OF SYMBIONIN, AN OLIGOMERIC PROTEIN SPECIFICALLY PRODUCED BY AN APHID ENDOSYMBIONT *in vivo*

H. Ishikawa

Zoological Institute, Faculty of Science, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan

While the primary endosymbiont of the pea aphid, *Acyrthosiphon psium* is capable of synthesis of more than several hundred proteins when taken out of the mycetocyte and incubated *in vitro*, it synthesizes only one protein, symbionin, when present inside the host cell. Since symbionin is not found among the proteins synthesized by the endosymbiont *in vitro*, it can be a key protein in order to maintain this symbiotic system stably and harmoniously. On 2D gel electrophoresis symbionin is an acidic protein with a molecular mass of 63 kDa, electron micrographs of negatively-stained molecules strongly suggested that native symbionin is a 14 subunit homo-oligomer composed of two stacked rings of 7 subunits

Immunological reactivity and N-terminal amino acid sequence analysis suggested that symbionin is highly homologous to the *E. coli* groEL protein, a heat-shock protein essential for cell viability and the assembly of bacteriophage capsids. The groEL protein is a member of chaperonins that include the Rubisco subunit-binding protein of chloroplasts and a mitochondrial heat-shock protein hsp60 required for the assembly into oligomeric complexes of polypeptides imported into the mitochondrial matrix. These results suggest that the selective synthesis of symbionin is important for the endosymbiont not only to endure the stress imposed by the intracellular conditions but also for a scaffold to stabilize macromolecular assembly processes essential for importing macromolecules into the endosymbiont.

CECROPIN-LIKE ACTIVITY OF THE GREATER WAX MOTH PUPAE UNDER INFLUENCE OF IMMUNOSUPPRESSIVE AGENTS

J. Jan

Insect Pathology Research Unit, Maria-Curie Sklodowska University, 20-033 Lublin, Poland

If pupae of the greater wax moth are injected with non-self particulate or soluble molecules they respond by producing three classes of antibacterial immune proteins: attacins, lysozyme and cecropins. Acidic electrophoresis of immune haemolymph followed by an antibacterial assay on the gel clearly revealed the presence of these three octivities of bactericidal factors in haemolymph from pupae but not from larvae of the moth. Injections of live *Enterobacter cloacae* or *Escherichia coli* induce a high level of bactericidal activities, whereas the response is markedly weaker after an injection of Weevers solution. The induction of antibacterial activity in pupal blood requires the selective synthesis of certain types of RNA responsible for immune proteins.

Inhibition of transcription by actinomycin D(0.03 μg per pupa) at different time after injection of the inductor showed that about 6 h are required for the formation of mRNA needed for the synthesis of bactericidal peptides of cecropin-like family. The induction of cecropins in insect haemolymph was also completely suppressed for 6-8 h by injection of cycloheximide at a concentration of 2.5 μg, an inhibitor of protein synthesis in eukaryotic systems. In general, the immune response was unaffected by injection of amethopterin or 5-fluorouracil. Other immunosuppressive agents of mammalian type such as cyclophosphamide or glycocorticosteride, hemisuccinate given once into insect hemocoela at lag phase of the induction at a sublethal dose only slightly, if at all, could inhibit the expression of cecropins activity in immunized pupae of the insect.

CONSERVATION OF 90 kDa POLYPEPTIDE IN IXODID TICK SALIVARY GLANDS AND CEMENT CONE

D.C. Jaworski,[1] R. Rosell,[2] L.B. Coons,[2] and G.R. Needham[1]

[1] Department of Entomology, Acarology Laboratory, The Ohio State University, 484 West 12th Ave., Columbus, OH 43210
[2] Department of Biology, Memphis State University, Memphis, TN 38152

Using a monospecific antiserum (12C) raised to a 90 kDa immunogenic component of *Rhipicephalus appendiculatus* glands, similar polypeptides from the salivary glands of *Dermacentor variabilis* and *Amblyomma americanum* were identified. However, several other bands were strongly reactive below 90 kDa in *D. variabilis* glands. Additionally, this antiserum labeled the cement cones of *D. variabilis*, *A. americanum* and *R. sanguineus*. It appears that the most reactive polypeptides in the cement cone were located near 70 kDa. This suggests

that the salivary gland polypeptide as it is found in the cement is modified. Immunostaining of the salivary gland of unfed and partially fed female *D. variabilis* localized the 12C antiserum in the d- and e- cells of the Type III acini. The amount of label found in the granules of unfed glands was visually greater than that seen in the granules of partially fed glands, so it appears that this component is secreted early in the feeding process. Collectively, these data suggest that the 90 kDa component of saliva is conserved among ixodid tick genera, and that a portion of the 90 kDa salivary gland polypeptide is deposited in the cement cone of ixodid ticks.

A NEW METHOD OF DETECTING HORMONE-BINDING PROTEINS ELECTROBLOTTED ONTO GLASS FIBER FILTER

L.S. Jefferies, and P.E. Roberts

Department of Entomology, (and Cell and Molecular Biology), Colorado State University, Fort Collins, CO 80523

We have identified juvenile hormone (JH) binding proteins by using a new method that allows electrophoretically separated proteins to bind JH after they are electroblotted onto glass fiber filter. In the two-striped grasshopper, *Melanoplus bivittatus*, a number of proteins are involved in the delivery of JH from its site of synthesis to the nuclei of fat body cells where it acts to induce vitellogenin synthesis. To identify JH binding proteins, hemolymph was separated on acryamide gels electroblotted onto glass fiber filters and incubated in [10-^{3}H] JH III. The amount of hormone bound by blotted proteins increased with the amount of protein on the filter, was competitively displaced by excess non-labeled hormone, and was affiliated with individual bands on fluorograms of proteins blotted after electrophoretic separation. Glass fiber filters etched with trifluoroactic acid was better than nitrocellulose, zeta probe, cellulose acetate or unetched filters. Phosphate (pH 6.0-7.3) or tris buffers (pH 7.3-8.0) worked equally well for the procedure. Unbound hormone was easily removed by short washes in buffer, and adequate binding for detection was achieved in a 15 min incubation. Preliminary data suggest that this technique may also be used to detect receptors, carriers, and binding proteins of steroid hormones. We are currently using electroblot incubation to monitor JH binding protein purification by anion exchange high performance liquid chromatography. (Supported by NIH AI-18470 and NSF DCB-8710056 to P.E.R. Preliminary studies supported by Biomedical Research Support Grants and Faculty Research Grants through Colorado State University.)

PEROXISOMAL ACYL-COA OXIDASE AND CATALASE ACTIVITY IN THE VENTRICULUS OF THE HONEY BEE, *Apis mellifera*

D.R. Jimenez, and M. Gilliam

USDA-ARS, Carl Haydn Bee Research Center, Tucson, AZ 85719

Peroxisomes are small, ubiquitous organelles that characteristically contain several oxidative enzymes and catalase. Their metabolic purpose varies in different tissues and is dependent on the complement of inducible enzymes within the organelles. Although peroxisomes are recognized as essential components in the cellular economy of microorganisms, plants, and animals, little is known about their function in insects. Using ultrastructural cytochemistry, we localized the peroxisomal marker enzymes, L-α-hydroxy acid oxidase (HAO) and catalase, within columnar cells of the honey bee midgut. Although HAO is found in many peroxisomes, its physiological role in organisms lacking the glyoxylate cycle remains obscure. Biochemical assays for glyoxylate cycle enzymes and uricase were negative in post-nuclear supernatant fluids from honey bee midgut tissue, but peroxisomal acyl-CoA oxidase (ACO)

activity was detected. ACO is a H_2O_2-producing oxidase that regulates peroxisomal β-oxidation of long chain fatty acids. We measured ACO and catalse activity in midgut tissues from free-flying worker bees of known ages. ACO activity peaked in young honey bees when pollen intake, midgut protein, and tissue weight were highest. As bees aged, and their diet shifted from pollen to carbohydrate, ACO activity declined indicating that this enzyme is involved primarily in the oxidation of fatty acids absorbed from pollen. Catalase activity increased as the bee aged, reaching its highest level in 35-day-old bees. Results from our ultrastructural and tissue fractionation studies indicate that peroxisomal metabolism is a crucial component in honey bee metabolism and eventual senescence.

THE IMMUNE RESPONSE IN *Trichoplusia ni* CHALLENGED WITH BACTERIA OR BACULOVIRUSES

H. Johansson, D. Andersons, H. Gunne, M. Hellers, and H. Steiner

Department of Microbiology, University of Stockholm, S-106 91
Stockholm, Sweden

The expression and processing of attacin and cecropin, two proteins mediating hormonal immunity in insects has attracted our interest. In order to study these processes we have expressed the proteins with a baculovirus vector in living larvae of *Trichoplusia ni.* The larvae, however, also respond to injections of foreign material with a set of endogenous immune proteins. We now report some basic facts about this immune response in *T. ni* and try to relate the induced proteins to the know immune proteins for *Hyalphora cecropia*.

The antibacterial response was elicited most effectively by live bacteria, however, an injection of wild-type *Autographa califonica* NPV also caused an induction. In contrast, no antibacterial response was observed when the virus was given *per os*. A suppression of the phenol oxidase activity was noted in larvae injected with virus.

Hemolymph form larvae induced with bacteria was fractionated electrophoretically and the gel was probed both with a bacterial overlay and Western blotting. There were 2-3 antibacterial bands that cross-react with anti-cecropin antibodies. One band contained and attacin like protein that could be distinguished from the cecropia attacin that we have expressed in the larvae. lysozyme activity was also induced.

INSECT IDENTIFICATION USING DNA PROBES

D.W. Johnson and A.F. Cockburn

Department of Entomology and Nematology and Insects Affecting Man and Animals Research Laboratory, USDA, University of Florida, Gainesville, Fl 32604

Using the four morphologically indistinguishable species of the *Anopheles quadrimaculatus* complex, we have shown that species-specific repetitive DNA probes can be isolated from mosquitoes and used for taxonomic identification of adults, larvae, pupae, or isolated body parts. Cloned species-specific repetitive DNA sequences hybridize to DNA from the species used to construct the library, but not to DNA from two closely related species. Mosquitoes or their parts are squashed between two membrane filters (squash blots), providing targets which can be hybridized to different probes to identify individuals. Freshly killed insects provide stronger signals than dried individuals, but a signal is obtainable from dried adults.

We have coupled a non-radioactive detection system (enzymatic detection of antibody bound to digoxigenin-tagged probe DNA) with the squash-blot technique. This non-radioactive

system also can be used to identify individual mosquitoes; however, radioactive detection is currently more sensitive. We are investigating the use of other detection techniques.

JUVENILE HORMONE REGULATION OF A METAMORPHOSIS-ASSOCIATED PROTEIN

G. Jones, M. Wozniak, and M. Manczak

Department of Entomology, University of Kentucky, Lexington, KY 40546

A protein has been identified which is highly expressed during larval-pupal metamorphosis of *Trichoplusia ni*, and the expression of which is sensitive to the presence of juvenile hormone. Immunological analysis following denaturing gel electrophoresis confirms that appearance of the protein in the hemolymph is due to new protein, and not due to modification of pre-existing hemolymph proteins. Additional experiments demonstrated that sensitivity of the appearance of the protein to juvenile hormone involves a transcriptional level of regulation. Northern analysis with cloned cDNA for the protein confirmed the involvement of transcriptional regulation. Additional molecular analysis of regulation of the expression of the protein will be presented. (Supported by NIH DK 39197.)

REDIRECTION OF HOST GENE EXPRESSION BY PARASITIC INSECTS

D. Jones, R. Farkas, T. Taylor, J. Chelliah, and D. Moore

Department of Entomology, University of Kentucky, Lexington, KY 40546

Parasitic wasps of the genus *Chelonus* inject into the host regulatory material which redirect the host for 1) precocious initiation of metamorphosis, 2) suppressed prepupal ecdysteroid production and 3) suppression of the immune response. Researchers have yet to identify materials that will induce in any insect complete precocious expression of genes which normally lead toward metamorphic commitment. They also have yet to identify material which, when injected into insect eggs, will suppress prepupal ecdysteroids without affecting ecdysteroid production during the previous larval-larval molts. However, the material injected by *Chelonus* wasps reliably causes both effects. We have been examining the biochemical and molecular basis of these effects and have identified a number of unique translation products associated with the parasitized condition. We have also identified cDNA clones encoding transcripts induced in host larvae. These results, and cloning of venom proteins associated with immunosuppressive effects, will be presented.

HEAT SHOCK PROTEINS IN *Sarcophaga crassipalpis*: DEVELOPMENTAL AND TISSUE-SPECIFIC EXPRESSION AND RESPONSE TO COLD SHOCK

K.H. Joplin, and D.L. Denlinger

Department of Entomology, The Ohio State University, Columbus, OH 43210

We have examined the heat shock protein (hsp) expression in the flesh fly *Sarcophaga crassipalpis*. Heat shock protein response in *Sarcophaga* is more complex than reported in *Drosophila*. 1) Hsps are induced at higher temperatures (40 to 43°C) in *Sarcophaga* than in *Drosophila*. 2) Heat-shocked tissues express two hsps, 65 and 72 MW, which are immunologically related to the *Drosophila* hsp 70 family. 3) Hsp expression is tissue specific in 3 day old *Sarcophaga* adults. Hsp 72 is found in the brain and integument while hsp 65 is

found in the terminalia and flight muscle. 4) Terminalia switch hsp expression from 72 to 65 after the first day of adult eclosion. 5) Another developmental switch between hsp 65 and 72 occurs at pupariation. Brain and integument express hsp 65 as wandering larvae but hsp 72 is expressed after pupariation.

Both heat and cold stress induce thermotolerance and recovery from cold shock induces expression of hsps in *Sarcophaga*. 1) Hsp expression is enhanced by increased intensity of cold treatment. 2) Brain and integument express hsps during the first hour of recovery from -10°C treatment. 3) A different set of proteins is expressed in the integument, but not in the brain, during the second hour of post-treatment labeling.

Identification of complex controls on the expression of hsps in *Sarcophaga* will allow the exploration of molecular control of gene function in this model system.

HORMONAL CONTROL OF PHEROMONE BIOSYNTHESIS IN THE REDBANDED LEAFROLLER MOTH

R.A. Jurenka, and W.L. Roelofs

Department of Entomology, Cornell University, New York State Agricultural Experiment Station, Geneva, NY 14456

The physiological mode of action of a brain hormone that controls pheromone biosynthesis in the redbanded leafroller moth (RBLR), *Argyrotaenia velutinana*, was investigated. Decapitated females exhibit a decrease in pheromone titer; however, when only the ventral nerve chord was severed pheromone titers remained high. These results indicate that the brain hormone, termed pheromone biosynthesis activating neuropeptide (PBAN), is released into the hemolymph and moves to the target tissue. PBAN has been isolated and identified from *Heliothis zea* and synthetic PBAN mimics the action of the brain hormone in RBLR. Synthetic PBAN (4 pmol) injected into decapitated females causes a ten-fold increase in pheromone titer. Isolated abdomens from decapitated females incubated on a 5 μl drop of saline containing PBAN (4 pmol) exhibited a ten-fold increase in pheromone titer. The incorporation of [1-^{14}C]acetate into the pheromone was also monitored using the isolated abdomen culture technique. Again a ten-fold increase in ^{14}C-acetate incorporation was observed after incubation with 4 pmol of PBAN. Selected removal of tissues in isolated abdomens indicated that the ventral nerve chord and the terminal abdominal ganglion are not involved in the action of PBAN. Removal of the corpus bursae indicates that this tissue has an inhibitory effect on pheromone biosynthesis but PBAN overcomes this inhibition. Pheromone biosynthesis was compared using *in vitro* and *in vivo* culture techniques.

HORMONAL AND PHEROMONAL REGULATION OF VITELLOGENIN SYNTHESIS IN HONEY BEES

H. Kaatz, and F. Dittrich

Department of Developmental Biology, University of Tübingen, D-7400 Tübingen, FRG

In the course of honey bee queen ontogenesis, vitellogenin, the yolk protein precursor first appears in the hemolymph of pharate adult honey bees. Decapitation and allatectomy experiments on pupae of different stages of development did not inhibit the production of vitellogenin. In other words, the initiation of vitellogenin synthesis in the imaginal fat body is independent of juvenile hormone and factors from the brain. Later on in adult development, the rate of vitellogenin synthesis in queens is modulated with the actual rate of egg production. Questions concerning the hormonal regulation of vitellogenin synthesis have been examined by culturing fat bodies *in vitro*. Head extracts of egg-laying queens added to

culture medium result in a 2.5 fold increase of vitellogenin synthesis by the explanted fat bodies. However, neither juvenile hormone, known to control vitellogenin synthesis in most insects, nor ecdysterone affect vitellogenin synthesis. Therefore, humoral factor(s), other than juvenile hormone modulates vitellogenin synthesis in honey bees. Additionally, queens inhibit vitellogenin synthesis in worker honey bees partially by a pheromone of their mandibular glands. In queenless workers--whether egg-laying or not--vitellogenin synthesis is elevated two-fold compared to queenright workers. This demonstrates that vitellogenin synthesis in honey bees also depends on the social situation.

HUMORAL IMMUNITY IN TSETSE

G.P. Kaaya

ICIPE Research Center, P.O. Box 30772, Nairobi-Kenya

Immunization of tsetse with sublethal doses of live bacteria conferred protection against subsequent lethal doses. Electrophoresis of immune hemolymph in SDS-PAGE revealed a remarkable increase in 2 proteins possessing M_r of 17 and 70 kDa, and the hemolymph inhibited growth of bacteria in agar cultures. Three classes of antibacterial proteins: attacins, cecropins and lysozyme were detected in the immune hemolymph. Lysozyme was present at low levels in the hemolymph of non-immunized tsetse, but increased significantly after immunization reaching peak 2 hrs later and both live and killed bacteria stimulated its production although live bacteria was more effective. No anamnestic response was observed in tsetse given secondary bacterial injections. Attacins and cecropins were not present in the hemolymph prior to immunization but appeared 6 hr and reached peak 24-48 hr post immunization and were stimulated only by live and not by killed bacteria. Injection of Cycloheximide, an inhibitor of protein synthesis in eukaryotic organisms inhibited production of both attacins and cecropins, suggesting that they are proteins, and that they are synthesized *de novo*, but increased the production of lysozyme. Inhibitor A, a protease from *Bacillus thuringiensis* also inactivated tsetse immune proteins. Female tsetse mounted a stronger response than males and it increased with aging in both sexes from emergence to about two weeks after which it declined gradually. Immunization of tsetse with *Escherichia coli* had no effect on development of *Trypanosoma brucei*, and injection of live trypanosomes failed to stimulate immune proteins. Furthermore, there was no difference in response between the trypanosome-infected and the non-infected tsetse.

SYNTHESIS OF HIGH SPECIFIC ACTIVITY TRITIATED 7-DEHYDROCHOLESTEROL AND CONVERSION STUDIES IN *Locusta migratoria*

C. Kappler,[1] F. Dollé,[2] C. Hetru,[1] B. Luu,[2] J. Hoffmann[1]

[1] Laboratoire de Biologie Générale, UA 672 du CNRS "Endocrinologie et Immunolgie des Insectes", 12 rue de l'université, Strasbourg, 67000, France
[2] Laboratoire de Chimie Organique des Substances naturelles, UA 31 du CNRS, 5 rue Blaise Pascal, Strasbourg, 67000, France

The last steps of ecdysone biosynthesis are now well characterized in *Locusta migratoria* and consist in three hydroxylations catalyzed by cytochrome P-450-dependent monooxygenases: the C-25 hydroxylation of 2,22,25-trideoxyecdysone followed by C-22 hydroxylation and finally C-2 hydroxylation.

We have now extended our studies to the first steps in ecdysone biosynthesis and, postulating that 7-dehydrocholesterol is involved in these first steps, we have synthesized this

Locusta migratoria into conjugated ecdysone and 2-deoxyecdysone, the incorporation rates being 10 times faster than those for cholesterol under the same conditions. *In vitro* incubations of 7-dehydrocholesterol with a 1000g supernatant of a follicle cell homogenate have occasionally yielded conversion rates into ecdysone which are as high as 10%.

EXPRESSION AND CHARACTERIZATION OF THE CHICK NICOTINIC ACETYLCHOLINE RECEPTOR (α-SUBUNIT) AND *E. Coli* ß-GALACTOSIDASE IN TWO INSECT CELL LINES USING BACULOVIRUS VECTORS

L. King,[1] A. Atkinson,[1] S. Mann,[1] A. Lawrie,[1] S. Mulshaw,[1] L. Obosi,[1] D. Beadle,[1] and F. Earley[2]

[1] School of Biological & Molecular Sciences, Oxford Polytechnic, Oxford, OX3 OBP
[2] ICI Agrochemicals, Jealotts Hill Research Station, Bracknell, RG12 6EY, U.K.

Replication of the baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV) was studied in two insect cell lines; the widely used *Spodoptera frugiperda* and also a *Mamestra brassicae* cell line. Evidence from virus titres (pfu/ml), the numbers of polyhedral inclusion bodies produced and SDS-PAGE analysis of infected cell proteins (in particular polyhedrin), suggests that AcNPV produces more polyhedra and polyhedrin protein in the *M. brassicae* cell line. Concurrent with the increased synthesis of polyhedrin protein is a reduction in the titre of extracellular virus. It therefore appears that the bi-phasic replication cycle of AcNPV is biased towards polyhedra formation in the *M. brassicae* cells and extracellular virus synthesis in the *S. frugiperda* cells. We have also compared levels of synthesis of a number of foreign proteins, expressed under control of the polyhedrin promoter in recombinant AcNPVs. In most cases expression levels of the foreign protein were three to four times greater in the *M. brassicae* cell line. The data presented show the results obtained with expressing the *E. coli* ß-galactosidase and chick nicotinic acetylcholine receptor (α-subunit) genes. Expression levels were monitored by SDS-PAGE and ONPG assay for ß-galactosidase and SDS-PAGE, Western Blotting with [^{125}I]α-bungarotoxin and toxin filter binding assays for the receptor subunit.

SEQUENCES RELATED TO IMMUNE PROTEINS IN THE MOSQUITO, *Aedes aegypti*

T. Knapp and J. Crampton

Wolfson Unit of Molecular Genetics, Liverpool School of Tropical Medicine, Liverpool L3 5QA, U.K.

We are interested in understanding the molecular aspects of insect vector-parasite relationships. The insect immune system may play some role in this relationship. We have therefore initiated a study aimed at defining and characterizing the genes in the mosquito, *Aedes aegypti*, which code for proteins related to the known immune proteins defined in other insects.

Cloned sequences related to the immune proteins attacin, cecropin, and diptericin have been isolated from an *A. aegypti* genomic DNA clone bank using synthetic oligonucleotides based on previously published data derived from other insect systems. Clones corresponding to the attacin-like, cecropin-like and diptericin-like sequences have been subcloned into a variety of vectors to aid further investigation. The subclones have been mapped using restriction enzymes. Using these maps, regions of the clones which hybridize to corresponding oligonucleotides have been subcloned into a variety of vectors to aid further investigation. The subclones have been used to probe northern and Southern blots of a variety of RNA and

genomic DNAs in order to define the number and organization of the genes coding for these proteins and the nature of their transcripts. Partial sequence data for the cecropin and diptericin clones has now been obtained.

Subsequently, these clones will be used to localize the genes for these putative immune proteins by *in situ* hybridization to chromosome preparations. It may also be possible to study the inducibility of these proteins by characterizing any differences in protein levels between *A. aegypti* strains refractory and susceptible to filarial parasites after infection.

CONSTRUCTION OF A NEW BACULOVIRUS VECTOR SYSTEM OF THE SILKWORM, *Bombyx mori*

J. Kobayashi,[1] H. Inoue,[1] I. Katoh,[2] Y. Yoshinaka,[2] Y. Ikawa[2]

[1] National Institute of Sericultural and Entomological Science Tsukuba, Japan
[2] Tsukuba Life Science Center, The Institute of Physical and Chemical Research, Tsukuba, Japan

Baculovirus vector systems have been established and used for high level expression of foreign genes. Among them, the vector system constructed from *Bombyx mori* nuclear polyhedrosis virus (BmNPV) is the only one that can express foreign genes *in vivo* on a large scale, mainly because of the agricultural background of the silkworm.

Recently, we established a new continuous cell line from embryos of the Japanese silkworm strain "Kuroko". One of the clones, NISES-BoMo-15AIIc, was proved to be susceptible to BmNPV and to produce many polyhedra in infected nuclei. The culture medium is MGM-448 (Mitsuhashi, 1984), supplemented with 10% fetal bovine serum (FBS). The population doubling time was about 3 days at 25°C. As BoMo-15AIIc cells adhere strongly to the surface of flasks, cells are suspended by pancreatin treatment for subculture.

The wild type BmNPV was isolated from infected larvae and purified by plaque assay using BoMo-15AIIc cells. DNA from one of the clones, BmNPV-P6E, was studied by restriction analysis with a synthesized 30 base long oligomer as hybridization probe, including a part of the conserved sequence found near the transcriptional start site of the polyhedrin gene. Results of the DNA analysis accompanied with sequencing data demonstrated that the polyhedrin gene of BmNPV-P6E was located in a 4.6 kb *Hind*III-*Eco*RI fragment. Several transfer vectors were constructed from BmNPV-P6E and expression of foreign genes by this new system is now under investigation.

MOLECULAR ANALYSIS OF ECDYSONE RESPONSE IN *Drosophila melanogaster*

M. Koelle, W. Talbot, M. Bender, and D.S. Hogness

Department of Biochemistry, Stanford Medical Center, Stanford, CA 94305

We are interested in understanding the molecular details of ecdysone response in *Drosophila*. Ashburner et al (1976, in: "Insect Development" P.A. Lawrence, ed., pp 203-225, Wiley, New York) have shown through studies of ecdysone induced chromosome puffing that ecdysone triggers a complex response in which the expression patterns of several classes of genes are differentially controlled. Our goal is to isolate the regulatory molecules involved in governing this response, and to test and extend Ashburner's model for ecdysone response at the molecular level. Towards this end we have cloned the *Drosophila* ecdysone receptor. We have also cloned two primary ecdysone inducible genes, call E75 and E74, which Ashburner's data predicted would encode protein(s) controlling the later stages of ecdysone response. We

have investigated the structure of these genes, their spatial and temporal patterns of expression, and have isolated mutations for each of the three genes.

The sequence of the ecdysone receptor gene predicts a 94 kDa protein homologous to the vertebrate nuclear receptor family. It is expressed at a detectable level throughout development with a strong peak of expression at the end of third instar. By antibody staining we see the protein localized in the nuclei of all tissues at all stages so far examined. We have examined the DNA binding specificity of this protein both *in vivo* and *in vitro*. In the *in vivo* assay we stain polytene chromosomes that are undergoing ecdysone response using anti-ecdysone receptor antibodies. As predicted by Ashburner, we see the ecdysone receptor bound at the sites of "early" ecdysone induced chromosome puffs, which are thought to contain genes whose expression is induced directly by the receptor. The ecdysone receptors are also seen bound at "late" puff loci, consistent with Ashburner's observation that these puffs are transiently repressed by ecdysone. *In vitro* we have shown that the receptor binds specifically to a 34 bp sequence that acts as an ecdysone response element, in that it can confer ecdysone inducibility on a normally ecdysone non-responsive promoter.

The E75 gene encodes two protein products which surprisingly, like the ecdysone receptor, have homology to the vertebrate nuclear receptors. Using an *in vivo* DNA binding assay similar to that for the ecdysone receptor, we see E75 protein bound to both early and late puff loci, consistent with this protein playing a role in regulation of these ecdysone induced puffs. The E74 gene encodes a protein which also binds DNA, and is homologous to the *ets-2* oncogene.

DIFFERENTIAL EFFECTS OF LIMITING POLYAMINE SYNTHESIS ON EXPRESSION OF RNAS DURING VITELLOGENESIS IN THE MOSQUITO *Aedes aegypti*

P.H. Kogan

Department of Entomology, Cornell University, Ithaca, NY 14853

Vitellogenesis in the mosquito, *Aedes aegypti*, is a complex process that requires the presence of specific molecules during specific times. In addition to the well recognized involvement of nutrients and hormones, another requirement for egg development is an increase in polyamines; ubiquitous, short carbon-chain cations necessary for growth and optimal macromolecular synthesis. Earlier work by this investigator has shown that inhibition of polyamine synthesis limits vitellogenin production and the increase in total RNA normally seen after a blood meal, which initiates egg production. Other researchers, primarily working will cell cultures, have evidence that blocking polyamine synthesis may have differential effects on expression of various RNAs. Vitellogenesis, with its precise timing of substantial macromolecular synthesis and concurrent increases in polyamines, is a good system to further investigate the interaction of polyamines and RNA expression in a whole animal model.

Two messenger RNAs expressed only during vitellogenesis in *A. aegypti* are vitellogenin (Vg) and a follicle specific product (FSp). Actin is constitutively expressed before and after blood feeding. The increase in total RNA seen after blood feeding is expected to include an increase in ribosomal RNA. Probes made for 18S rRNA and the three messages listed above were used to determine if inhibition of polyamine synthesis during vitellogenesis would affect all RNA expression equally. While messenger RNA for actin does not appear to be affected at all, the Vg and the FSp RNAs are clearly reduced, possibly with a greater relative decrease of the FSp message. The addition of polyamines to mosquitoes treated with inhibitor can reverse the inhibitory effects.

AN *in vitro* STUDY OF VITELLOGENIN ENDOCYTOSIS BY OOCYTES OF THE MOSQUITO, *Aedes aegypti*

C.N. Koller, T.S. Dhadialla, and A.S. Raikhel

Department of Entomology and Program in Cell and Molecular Biology, Michigan State University, East Lansing, MI 48824

An *in vitro* assay was used to study the uptake of vitellogenin (VG) by oocytes of the mosquito, *Aedes aegypti.* The VG, obtained from the culture of fat bodies *in vitro*, was metabolically labelled to a high specific activity (3 to $6x10^5$ CPM/μg) using [^{35}S]methionine. Purification of both VG and vitellin (VN) from ovaries was achieved with ion-exchange chromatography on DEAE-Sepharose CL6-B. In an optimized *in vitro* system, mosquito oocytes in intact ovaries maintained stable endocytotic activity for at least 4 h. The rate of VG uptake was maximal at pH 7.5. It declined at acid pH and ceased abruptly at pH 6.3. Accumulation of VG by mosquito oocytes demonstrated features of receptor-mediated endocytosis; i.e., temperature dependence, saturability, selectivity, and tissue specificity. The uptake of VG was inhibited at 4°C with only 6% of uptake achieved at 27°C. At 24 h post-blood meal, uptake of VG by oocytes at 27°C neared saturation with a VG concentration in the medium of 8 μg/μl. Saturation kinetics generated for VG endocytosis by these oocytes produced a V_{max} = 3.2 μg/μl/h and an apparent K_{uptake} = $8.4x10^{-6}$M. Ovaries accumulated 10 times the amount of VG compared to mouse IgG, while uptake of VN was 71.6% of that for VG, indicating that oocytes of *Aedes* are able to distinguish these proteins. Non-ovarian tissues, fat body and Malpighian tubules, accumulated both VG and IgG at equally low levels. During the vitellogenic cycle, the rate of VG uptake by oocytes showed a steep and linear increase between 6 and 24 h post-blood meal. The peak of VG uptake occurred between 24 and 30 h post-blood meal, followed by a precipitous decline and cessation of uptake by 36 h.
(Supported by NIH grant HD-22958.)

SPECIAL VARIANT OF SOMATIC MYOGENESIS OF *Bombyx mori* L. LARVAE

S.A. Komarov

Institute of Cytology of the Academy of Sciences of the USSR, Leningrad, USSR

With the help of EM and EM-autoradiography with ^{3}H-thymidine and cytophotometry we have shown that differentiation of muscle fibers in intersegmental growing muscles of *B. mori* larvae did not inhibit DNA synthesis in muscle nuclei though totally blocked mitoses. DNA synthesis under inhibition of mitoses resulted in increase of ploidy level of the above nuclei. Mitotical activity was persisted only in myosatellites as it was originally revealed for the cells of this species. In the process of growth and development of muscle fibers in larvae both the ploidy level and the number of nuclei were shown to be increased. Increment of the number of muscle nuclei was suggested to be correlated with proliferation and the following fusion of myosatellites with muscle fibers, and probably with the fragmentation of the high polyploid muscle nuclei.

On the basis of the obtained data we have suggested the special variant of somatical myogenesis, and proposed its pattern.

EFFECT OF REARING TEMPERATURE ON THE STORAGE PROTEIN IN THE SILKWORM *Bombyx mori*

M. Krishnan,[1] and S. Mathavan[2]

[1] School of the Biological Sciences, Madurai Kamaraj University, Madurai 625 021, India
[2] Present address: c/o Dr. Susuma Maeda, Department of Entomology, University of California, Davis, CA 95616

Storage protein (SPL : 78 kDa, female specific. SP-2 : 74 and 68 kDa : two subunits) profile in the haemolymph and fat body of the silkworm *Bombyx mori* was detected following SDS-PAGE. The proteins were detected during the developmental period of the final instar reared in the experimental temperatures of 25, 30 and 35°C. Coomassie blue stained gel of the haemolymph of 24 h old larvae showed 12 polypeptides ranging from 100 to 18 kDa. Similar analysis of fat body or haemolymph of the larvae reared at different temperatures or larval period did not reveal a significant change in the number of polypeptides. However, the quantity of SP-1 proteins increased with increasing developmental period and reached the maximum prior to spinning. This process is faster at higher temperature when compared to low temperatures. Observation of SP-2 proteins in the haemolymph is similar to one observed for SP-1 protein. Quantity of SP-1 and SP-2 proteins in the fat body was found to be at low level during the larval period: it increased during prepupal period. The results are discussed with reference to the published data.

GENETICS OF P-450 MEDIATED PYRETHROID RESISTANCE IN THE MOSQUITO *Anopheles stephensi*

H. Ladonni, and H. Townson

Department of Medical Entomology, Liverpool School of Tropical Medicine, Liverpool L3 5QA, United Kingdom

Larval selection with permethrin was carried out on a pyrethroid tolerant strain of *An. stephensi* from the United Arab Emirates. Within three generations a highly resistant line was produced with a resistance ratio of >1000 at the LC_{50} compared with a wholly susceptible strain. Crosses and backcrosses between susceptible and resistant strains indicated that permethrin resistance in the selected line is controlled by a single major gene, which is autosomally linked.

Resistance behaves as an incompletely recessive character (dominance ratio = -0.34) and extends to other pyrethroid compounds. Piperonyl butoxide has a marked effect on permethrin resistance, with a synergist ratio of 426 in the selected line. This indicates that the major effect of the resistance gene lies in the control of a mixed function oxidase enzyme belonging to the cytochrome P-450 multigene family. In a related poster, Gerke, Bonet, Crampton and Townson describe preliminary data from molecular cloning experiments of an elevated level of a P-450 transcript in the pyrethroid resistant strain.

cDNAS FROM NEUROSECRETORY CELLS OF BRAINS OF *Locusta migratoria* ENCODING A NOVEL MEMBER OF THE SUPERFAMILY OF INSULINS

M. Lagueux, L. Lwoff, M Meister, F. Goltzené, and J.A. Hoffmann

Laboratoire de Biologie Générale, UA 672 du CNRS, "Endocrinologie et Immunologie des Insectes", 12 rue de l'Université, Strasbourg, 67000, France

A 5 kDa peptide has been isolated for neurohaemal lobes of the corpora cardiaca of adult females of *Locusta migratoria* and has been fully characterized, as reported in the accompanying presentation (Hietter, Van Dorsselaer, Green, Denoroy, Hoffmann, and Luu). We have synthesized oligonucleotide probes based on the peptide sequence and screened cDNA libraries prepared from the neurosecretory cell region of this insect. We will report the isolation of several cDNAs, the nucleotide sequence of which predict a large precursor molecule of 145 residues (15.7 kDa) containing the newly isolated 5 kDa peptide. Interestingly, on either side of this peptide we find regions homologous to the A and B chains of the insulin superfamily. In particular, the number and relative positions of the cysteins characteristic of the insulins are conserved in the *Locusta* molecule which we propose to call *Locusta* insulin related peptide (LIRP). Our results indicate that the 5 kDa peptide isolated from the corpora cardiaca of *Locusta* is a positional analogue of the C-peptide of insulins although its sequence shows no homology to any known C-peptide of the superfamily.

THE CONTROL OF MEIOTIC REINITIATION BY ECDYSONE IN *Locusta migratoria* OOCYTES: ACTION OF ECDYSONE INVOLVES A TRANSITORY DECREASE IN OOLEMMAL ADENYLATE CYCLASE ACTIVITY

R. Lanot, M.O. Sellam, J. Thiebold, and J.A. Hoffmann

Laboratoire de Biologie Générale, UA 672 du CNRS, "Endocrinologie et Immunologie des Insectes", 12 rue de l'Université, Strasbourg, 67000, France

We have previously demonstrated (Lanot et al., 1987, Develop. Biol. 121:174-181; Lanot et al., 1988, Develop. Biol. 126:212-214) that the reinitiation of meiosis in vitellogenic oocytes (prophase I) of *Locusta migratoria* is triggered by ecdysone produced in the follicle cell epithelium and present a series of data which point to the involvement of adenylate cyclase:
(1) - the intracellular concentration of cAMP decreases significantly (≈40%) in the oocyte at the time when meiotic reinitiation is induced;
(2) - drugs which are known to increase the intracellular concentration of cAMP antagonize the stimulatory action of ecdysone;
(3) - inhibitors of adenylate cyclase mimic the action of ecdysone;
(4) - ecdysone treatment of excised oocytes is followed by a decrease in intracellular cAMP;
(5) - ecdysone reduces the adenylate cyclase activity when added to oocyte plasma membrane preparations *in vitro*.

SYNTHESIS OF ANALOGS OF (Z)-9-TRICOSENE: PROBES FOR MONOOXYGENASE MECHANISMS IN THE HOUSEFLY

B. Latli,[1] and G.D. Prestwich,[1] R. Eppard,[2] G. Blomquist[2]

[1] Department of Chemistry, State University of New York, Stony Brook, NY 11794-3400
[2] Department of Biochemistry, University of Nevada, Reno, NV 89577

The cytochrome P-450 monooxygenase(s) present in house fly cuticle convert the pheromone (*Z*)-9-tricosene into 9.10-epoxide and to 14-keto metabolites. These reactions correspond to oxygen insertion into an alkene pi electron system and to oxygen insertion into an alkane C-H bond, respectively. Indeed, the ketone requires a second oxidation of C-H bond. Structurally, it is conceivable that both metabolites can arise by the action of a single monoxygenase, depending on the orientation of the substrate in the active site. To explore these possibilities, we synthesized a selection of compounds (and their expected metabolites) possessing modifications in the 14-position and in the alkyl chains of (*Z*)-9tricosene.

(1) Blocking the C-14 position with one methyl would allow observation of the expected alcohol intermediate in ketone production.
(2) Blocking the C-14 position with a fused cyclopropyl group or either mono- or difluoro substituents gives a compound resistant to oxidation at this site yet still capable of 9,10-epoxidation.
(3) Incorporation of a perfluorooctyl chain probes the importance of hydrophobicity of the alkyl chain and the nucleophilicity of the olefin on the epoxidation.
(4) Introduction of an additional alkene in the chain or an *exo* methylene at C-14 provides an alternative site of epoxidation.

We report the synthetic procedures and biochemical results for this study.

CHEMICAL INHIBITION OF THE PHEROMONE-GENERATING ALCOHOL OXIDASE OF FEMALE *Heliothis virescens* MOTHS

B. Latli, M.A. Handley, and G.D. Prestwich

Department of Chemistry, State University of New York, Stony Brook, NY 11794-3400

The abdominal pheromone gland of the female moths of the noctuid *Heliothis virescens* contain a robust cuticle-associated alcohol oxidase (AO). This enzyme converts primary alcohols to aldehydes, which are then released as a sex pheromone blend. Enzyme activity is conveniently measured in dried, macerated glands in organic solvents. A series of functional group analogs of the (*Z*)-11-hexadecenol were synthesized and their inhibition of AO activity was determined by a capillary GC assay. Incubations were conveniently performed in hexane solutions and the assay mixtures were analyzed directly. Of twelve analogs, only the thiol, primary amine, and cyclopropylamine analogs showed substantial inhibition of AO.

MOLECULAR CLONING AND SEQUENCING OF A cDNA FOR THE HEMOLYMPH JUVENILE HORMONE BINDING PROTEIN OF LARVAL *Manduca sexta*

K.A Lerro, and G.D. Prestwich

Department of Chemistry, State University of New York, Stony Brook, NY 11794-3400

The juvenile hormone binding protein (JHBP) from the hemolymph of fifth instar *Manduca sexta* larvae was purified using gel filtration chromatography, ion exchange chromatography, and preparative SDS-PAGE. Polyclonal rabbit antibodies were raised against this homogeneous protein, and high titer antisera were used to identify and isolate potential JHBP cDNAs from a fat body expression library in λ ZAP II. The fat body cDNA library was prepared from day one fifth instar larval polyA RNA, and the second DNA strand was formed using RNase H for RNA primer formation and reverse transcriptase prior to DNA polymerase I reaction. Eleven putative JHBP cDNA clones were isolated and subcloned into Bluescript plasmid; cDNA inserts were about 800 base pairs in length. Analysis of total protein from transformed *E. coli* XL1-Blue cells by protein blotting and immunodetection showed an immunoreactive protein as a ß-galactosidase fusion product at 36 kDa. This 36 kDa fusion protein, like the authentic 32 kDa JHBP, was specifically photoaffinity labeled with [^{3}H]-EHDA, clearly showing that JH binding capacity is present in the cloned protein. Both strands of one CDNA insert have been sequenced by the Sanger dideoxynucleotide method, and a translational product has been identified.

ISOLATION AND CHARACTERIZATION OF mRNA IN VITELLOGENIC AND NONVITELLOGENIC GRASSHOPPERS

Y. Li, and P.E. Roberts

Department of Entomology, Colorado State University, Fort Collins, CO 80523

Insect juvenile hormone (JH) stimulates the transcription of genes for vitellogenin and other proteins. To define the interaction of JH receptors with genes activated by JH, we isolated and characterized JH stimulated mRNAs from the two-striped grasshopper, *Melanoplus bivittatus*. Fat bodies of adult females have maximal production of vitellogenin on day 7 with a gradual decrease to day 17. Cytoplasmic RNAs were isolated from vitellogenic (day 8) and nonvitellogenic (day 0) female fat body by both phenol and guanidinium methods. It is evident that the guanidinium method gives undegraded RNA with high yield and good purity. Both day 8 and day 0 poly(A)$^+$ RNAs were selected by oligo(dT) cellulose columns. The molecular weights were determined on agarose gel or on polyacrylamide gel under denaturing conditions. Polyacrylamide gel electrophoresis of poly(A)$^+$RNA from day 8 shows the presence of a mRNA of about 7 kb, which is absent from poly(A)$^+$ RNA prepared from day 0 female fat body. cDNAs from this and other JH stimulated RNAs are being characterized. (Supported by the Colorado Agricultural Experiment Station).

DNA TRANSFECTION OF AN *Aedes aegypti* MOSQUITO CELL LINE

G. Lycett, P. Eggleston, and J. Crampton

Wolfson Unit of Molecular Genetics, Liverpool School of Tropical Medicine, Liverpool L3 5QA, U.K.

The genetic manipulation of an organism requires an understanding of processes such as gene recombination, expression and regulation. To study these phenomena in *Aedes aegypti* various techniques for introducing cloned DNA sequences into cultured cells have been attempted. Details and comparisons of these methods, including calcium phosphate, polybrene, lipofectin, and electroporation mediated gene transfer will be described.

The DNA used for transfection was a *Drosophila* P transposable element vector (Steller and Pirrotta, 1985, EMBO J. 4:167), with positive selection of transformants by G418 antibiotic resistance. Problems associated with these factors will be discussed, together with an outline of current analyses of transformed cell line DNA's and possible developments in DNA vector constructs.

DEVELOPMENT OF MONOCLONAL ANTIBODIES TO AKH AND PBAN SYNTHETIC PEPTIDES BY *in vitro* IMMUNIZATION.

M. Ma, C.-Q. Yu, M. Blackburn, and A. Raina

Department of Entomology, University of Maryland, College Park, MD 20742
Chemical Ecology Laboratory, USDA-ARS, Beltsville, MD 20705

We are reporting the successful development of monoclonal antibodies to *Heliothis* AKH and PBAN synthetic peptides by *in vitro* immunization followed by cell fusion. This protocol does not require the conjugation of small peptides to a larger carrier protein for improving their antigenicity. Balb/c mice were initially primed with 5 ug of synthetic peptide/mouse in complete Freund's adjuvant. After two weeks, splenocytes from each mouse were released into 10 ml of RPMI 1540 + FCS with 10% El-4 thymoma cell supernatant. 5 ug of synthetic peptide was also added for the induction of B-cell secondary response. After 3 days of *in vitro* immunization, the splenocytes were harvested and fused with FOX myeloma cells using Kodak 1450 PEG as fusant. The hybridomas were screened with an indirect ELISA using the synthetic peptides as coating antigen. 10 and 15 stable monoclonal antibody-secreting cell lines have been established for AKH and PBAN respectively. Western blot and immunohistochemical procedures were used to identify the poly-peptides bound to the monoclonal antibodies and the sites of hormone synthesis.

ENHANCEMENT ACTIVITIES OF FIBROIN AND SERICIN PROMOTERS IN *in vivo* ASSAY SYSTEMS OF THE SILKWORM, *Bombyx mori*

H. Maekawa,[1] H. Yamauchi,[2] N. Miyajima,[1] K. Okano,[1] K. Ohta,[1] N. Takada,[1] S. Takiya,[3] O. Ninaki,[4] Y. Kikuchi[5]

[1] National Institute of Health, Toyko 141, Japan
[2] Forest and Forest Production Research Institute, Japan
[3] National Institute of Basic Biology, Japan
[4] National Institute of Sericulture and Endocrinology Sciences, Japan
[5] Kyodo Shiryo Co., Japan

We assayed the promoter activity of fibroin and sericin genes in the cultured cell and silk gland of *Bombyx mori* by the chloramphenicol acetyltrasferase (CAT) activity. When the deleted promoters of fibroin gene were analyzed in the cultured cell, SES-Bm-130T derived from 10 day embryos of the strain 130, transfected by the calcium phosphate method, the regions until -31, -72 and -238 did not promote so much activity of the CAT. While it was indicated by the injection of the CAT genes containing those deleted promoters of the fibroin gene to the nucleus of the silk gland that the region up to -238 is necessary for the transcription of the fibroin gene. This evidence in the silk gland is partly consistent with the results of *in vitro* transcription system published elsewhere. The silk gland system is useful and convenient because one silk gland cell of the 4th instar larvae has the genome of 4 X 10^4 ploidy and corresponds to 2 X 10^4 cells of 2n. Furthermore, the more upstream region, up to about -5 Kb, of fibroin and sericin genes seems to be also important. We want to discuss more details.

THE ALPHA-GLUCOSIDASE OF *Aedes aegypti* SALIVARY GLANDS

O. Marinotti and A.A. James

Department of Molecular Biology and Biochemistry, University of California, Irvine, CA 92717

The salivary glands of the adult female *Aedes aegypti* are the final destination of most pathogens transmitted by this insect. Nevertheless, the lack of information about the normal mechanisms involved in control of protein synthesis, processing and secretion by this organ makes it difficult to understand the interactions between the insects and the parasites at the molecular level. In order to understand better these mechanisms, we have characterized one of the proteins synthesized by the salivary glands of the adult *Aedes aegypti*. This protein is an alpha-glucosidase that is able to hydrolyse maltose, maltriose and sucrose, but is not able to hydrolyse melibiose, melezitose, raffinose and trehalose. Furthermore, this is a soluble glycoprotein with molecular weight of 68,000, that is secreted by the salivary glands. Our analysis showed that this enzyme is present in at the same level in both males and females and that more than 80% of the total activity is localized at the proximal lateral lobes of the female salivary glands. The variation of alpha-glucosidase activity in the salivary glands of mosquitoes, kept under different diets will be presented and discussed. These data indicate that this alpha-glucosidase is the product of the gene *Maltase-like i* described previously by James et al. (Gene, 1989, 75:73-83)

ISOLATION AND CHARACTERIZATION OF A YELLOW-COLORED PROTEIN FROM THE HEMOLYMPH OF *Manduca sexta*

R.R. Martel and J.H. Law

Department of Biochemistry, University of Arizona, Tucson, AZ 85721

A yellow-colored protein (YCP) has been isolated from the hemolymph of fifth instar and wandering larvae of the tobacco hornworm, *Manduca sexta*. YCP has a molecular weight of approximately 32 kDa as determined by sodium dodecylsulfate polyacrylamide gel electrophoresis. Gel filtration chromatography of native YCP indicated that the protein is a monomer.

The N-terminal amino acid sequence of YCP was determined:
Ser-Lys-Asp-Ser-Val-Thr?/Ser?-Val-Asn-Gly-Lys-Asn-Tyr-Gly-Lys-Glu-Val-Leu-Lys-Asp-Asn-Ile-His-Gln-Ala-Tyr-Gln-Leu-Ser-Phe-Asp-Glu....

Analysis for carbohydrates has revealed that YCP is glycosylated. This glycosylation may contribute to the anomalous behavior of YCP during concanavalin A affinity chromatography. When sugar-free buffers are used, most *Manduca* hemolymph proteins are either excluded or retained by the concanavalin A matrix; in contrast YCP is retarded. The chromophore of YCP exhibits an absorbance maximum at 405 nm, giving the protein its yellow appearance. Experiments to identify this chromophore are in progress. YCP is synthesized by the fat body as demonstrated by the *in vitro* incubation of larval fat body in the presence of [^{35}S] methionine, followed by immunoprecipitation with anti-YCP, electrophoresis and autoradiography. In addition, immuno-blotting has indicated that the protein is present in the hemolymph of *Manduca sexta* at all stages of development and in both sexes.

STRUCTURE AND FUNCTION OF tRNA GENE OF MUTANT SILKWORM INHIBITING FIBSYNTHESIS

K. Matsuzaki and I. Oh'hara

National Institute of Sericultural and Entomological Science and Kinki University, School of Medicine, Tsukuba and Ibaraki and Osaka, Japan

Silk fibroin is synthesized in the posterior silkgland of the silkworm. Therefore, characterization of the posterior silkgland of the normal silkworm was made. In particular, it is clarified that the tRNA from the posterior silkgland consists of a specific one to the posterior silkgland and another one to the cell. In the case of the mutant silkworm symbolized as Nd and Nd-s, fibroin is hardly synthesized in the posterior silkgland. Furthermore, the tRNA from the Nd and Nd-s mutant did not indicate the presence of the silkgland specific tRNA in a large amount, although it appeared abundantly in tRNAs such as glycine tRNA and alanine tRNA of the posterior silkgland from the normal silkworm. In order to understand how alanine tRNA and glycine tRNA are controlled at the level of gene in Nd and Nd-s mutant, alanine tRNA gene and glycine tRNA gene were cloned and characterized by restriction and M13 method. Sequence glycine tRNA genes from the several organisms were compared. In this case sequences of 5´ flanking were different from each other except for TATA and CAAT structure. In sequences of 5´ flanking of glycine tRNA genes from mutant and the normal silkworm, both sequences were partially different from each other.

UTILITY OF RIBOSOMAL RNA SEQUENCE DATA FOR RESOLVING QUESTIONS IN INSECT PHYLOGENY

B.A. McPheron

Department of Entomology, Pennsylvania State University, University Park, PA 16802

The phylogenetic relationships among insect orders have remained obscure despite repeated attempts to unravel morphological homology. The net result of numerous studies has been an inability to resolve relationships among most of the orders. Recent application of molecular techniques to systematics has generated hope that sequence variation in some conservative molecule might provide insight into insect phylogeny. I have examined sequence variation in the small subunit ribosomal RNA (rRNA) molecule within the Endopterygota. Partial rRNA sequences were obtained from several orders in the Endopterygota using reverse transcriptase-mediated primer extension (five primers yielded approximately 1100 of 1900 bases in this molecule). Sequences were aligned using the Needleman-Wunsch algorithm, and these alignments were adjusted by eye with consideration give to the estimated secondary structure of the molecule. Different regions of the small subunit rRNA show substantial variation in the amount of sequence evolution that has taken place--some regions were difficult or impossible to align across all orders. Regions of uncertain alignment, including several areas with large insertion/deletion events, were excluded from the analyses. Bases at variable positions were used as characters in parsimony analysis (PAUP).

Multiple representations of several orders were sequenced and consistently cluster together in the most parsimonious trees. Parsimony analysis supports the previously accepted relationship among the panorpoid orders Diptera, Lepidoptera, Trichoptera, and Mecoptera. Sequence data place the Coleoptera with the Neuroptera and indicate an affinity between the Hymenoptera and the panorpoid orders.

MOTH TESTES ENGAGE IN PROTEIN TRAFFICKING: AN EXPOSE

S.G. Miller, R.F Leclerc, and S.-J. Seo

USDA/ARS Insect Attractants Laboratory, 1700 SW 23rd Drive, Gainesville, FL 32604

Three storage proteins of approximately equal abundance occur in the blood of *Heliothis virescens*. Antisera to these polypeptides having subunit molecular weights of 82, 76, and 74 kDa have been produced and we have also sequenced partial cDNA clones corresponding to the 82 and 76 kDa proteins. Immunoblot studies revealed that the 82 kDa protein (and to a lesser extent, the 74 and 76 kDa polypeptides) accumulates in testes, and the testis sheath was shown to be the site of synthesis based on immunoprecipitation and Northern analyses. Some of the newly-synthesized 82 kDa protein is deposited within sheath cells as granules and some is exported into the lumen of the testis. Immunocytochemical studies using the electron microscope revealed that the testicular fluid is taken up by pinocytosis into cyst cells which envelop the developing spermatids, and this material can then been seen to be transferred to the germ cells themselves. Transverse sections through elongated spermatids reveal storage protein accumulation within the mitochondrial derivatives, reflecting entrapment of testicular fluid during nebenkern formation. Three interesting conclusions are derived from these observations: (1) the blood-testis barriers does not exist at the level of the cyst cell as is generally thought; (2) novel functions for the storage proteins are suggested by the existence of a distinct intratesticular pool; and (3) future studies of insect spermiogenesis may be enhanced since any macromolecule (antibodies, plasmid DNAs, etc.) which can be introduced into the testis may be sequestered by sperm.

IDENTIFICATION OF A SPERM-SPECIFIC GroEL ISOFORM IN INSECTS

S.G. Miller, and R.F. Leclerc

USDA/ARS, Insect Attractants Laboratory, 1700 SW 23rd Drive, Gainesville, FL 32604

A protein complex containing a 63,000 Mr protein (p63) and a 60,000 Mr protein (p60) has been purified from sperm mitochondria of *Heliothis virescens*. This particle sediments at 16-18s on glycerol gradients and possesses a weak ATPase activity distinct from F_1. While mitochondrial preparations from all tissue types contain p60, the complex isolated from sperm is assembled from both p60 and p63 subunits. Immunoblot analyses revealed that p60 and p63 are structurally related, and further, that both are homologues of the *E. coli* groEL protein and eukaryotic "chaperonins". Sequencing of a genomic clone corresponding to p63 revealed a protein sharing significant amino acid sequence homology with both groEL (45%) and a human chaperonin (61%). By analogy with other well-characterized groEL-like proteins, the p60 and p63 protein complex serves a general role in the assembly of intramitochondrial protein subunits into functional structures. Two observations suggest that complexes containing p63 serve a functional role in mitochondrial biogenesis which is unique to sperm cells. First, the particle isolated from sperm exists as a single seven-member ring in contrast with proteins prepared from *E. coli* and lower eukaryotes which appear as double rings. And second, the p63 protein exists as a species-specific charge variant when analyzed by 2D gel electrophoresis. Expression vector-based functional tests of this notion are now possible with the availability of a full-length p63 clone.

PRELIMINARY EVIDENCE FOR HONEY BEE SPERM-MEDIATED DNA TRANSFER

C.P. Milne Jr., F.A. Eishen, J.E. Collis, and T.S Jensen

Department of Entomology, Washington State University, Pullman, WA 99164-6432

A recent report in *Cell* (1989, 57:717-723) found that sperm can be used as vectors to transfer foreign DNA into mouse eggs. Since honey bee semen can be easily collected and used to inseminate queens, we attempted DNA transfer with treated sperm. No honey bee promoters have been characterized and heterologous promoters have not been assayed for activity in bees. Therefore, we chose a 1 kb fragment from a commercially available plasmid that can be detected down to 10 fg in 1 μg of honey bee genomic DNA by the polymerase chain reaction with commercially available sequencing primers. Honey bee sperm was collected and incubated in a solution containing the 1 kb fragment for 1 hour. Virgin honey bee queens were instrumentally inseminated with this treated sperm and placed in small colonies for egg laying. DNA was extracted from worker larvae that developed from fertilized eggs laid by these queens. Results of the polymerase chain reaction of 1 μg of DNA samples from these larvae revealed the presence of the 1 kb fragment in about a third of the progeny.

EXOCRINE AND ENDOCRINE SIGNALS IN TERMITE CASTE DIFFERENTIATION

M.A. Mohamed,[2] and G.D. Prestwich[1]

[1] Deparment of Chemistry, State University of New York, Stony Brook, NY 11794-3400
[2] Honey Bee Breeding, Genetics, and Physiology Research Unit, 1157 Ben Hur Road, Baton Rouge, LA 70820

A molecular understanding of the control of cast differentiation remains one of the unsolved problems in developmental biology of social insects. Changes in the hemolymph juvenile hormone binding proteins (JHBPs) of the Eastern subterranean termite *Reticulitermes flavipes* occur during naturally or chemically-induced differentiation of workers to presoldiers. These subtle biochemical changes which precede visible physiological changes are detected by photoaffinity labeling of the JHBPs with [^{3}H]-EFDA followed by SDS-PAGE and fluorescence autoradiography. This assay has allowed (i) comparison of effects of several juvenoid IGRs *in vivo* and *in vitro*, (ii) determination of time course of JHBP changes in natural or methoprene-treated termites, and (iii) comparison of JHBPs of natural castes. In addition, we have demonstrated the existence of soldier- and nymph-produced primer pheromones which act to suppress methoprene-induced differentiation.

SELECTABLE MARKERS FOR TRANSFORMATION OF MOSQUITO AND MAMMALIAN CELLS

T.J. Monroe, J.O. Carlson, D.L. Clemens, and B.J. Beaty

Department of Microbiology, Colorado State University, Fort Collins, CO 80523

Because of the generality of the heat shock response in nature we investigated the *Drosophila* hsp70 promoter in both mammalian and mosquito cell lines and have shown that it can be used to transcribe selectable markers and other genes of interest when incorporated into DNA constructs. We have used this promoter to transcribe both *neo* and *hyg* antibiotic resistance gene and have stably transformed both BHK-21 cells and C6y/36 (*Aedes albopictus*) mosquito cells with these markers. We have also shown in these cells that hsp70 is not always an inducible promoter when incorporated into the cell genome an expression often occurs constitutively.

The isolation of stable transformants of mosquito cells will allow a number of interesting experiments to be done. Previous experiments have relied only on transient expression of the introduced DNA. The ability to use the same constructs in both mosquito and mammalian cells will allow comparative studies to be done in both systems. This will be of particular interest in studying expression of arboviral genes in these cells since both types of cells are capable of acting as host cells for virus replication.

RESTRICTION FRAGMENT LENGTH POLYMORPHISMS IN MIDDLE REPETITIVE DNA OF *Aedes albopictus* DETECTED BY SOUTHERN BLOT HYBRIDIZATION

T.J. Monroe, J.O. Carlson, and B.J. Beaty

Department of Microbiology, Colorado State University, Fort Collins, CO 80523

During the development of gene transfer systems in the mosquito, a moderately repeated DNA sequence was isolated and cloned from *Aedes albopictus* cells (clone C6/36). This 2 kb fragment was used as a probe in southern blot hybridizations of genomic DNA from several geographically isolated populations of *Aedes albopictus* mosquitoes. This DNA element was found to be highly polymorphic no only between populations but also between individuals of the same population. The element has been further characterized by restriction mapping, northern blot, and sequence analysis.

An element such as this has potential use in determining geographical origins of disease carrying insects as well as genetic marker for linkage studies. Sequence analysis is in progress and may elucidate the role of this repetitive element.

TRANSFORMATION OF THE MOSQUITO *Aedes aegypti* USING THE "P" ELEMENT, ISOLATED FROM *Drosophila melanogaster*

A. Morris, P. Eggleston, and J. Crampton

Wolfson Unit of Molecular Genetics, Liverpool School of Tropical Medicine, Liverpool L3 5QA, U.K.

The "P" element, a transposable genetic element isolated from *Drosophila melanogaster*, is being used as a DNA transformation vector in the manipulation of the genome of the mosquito *Aedes aegypti*. A micro-injection system has been developed for the introduction of DNA into the developing embryo. Genomic DNA analysis of surviving adults and their progeny has revealed the integration of injected DNA into the chromosomes of these individuals. There is growing evidence that these events are not mediated by transposition, although the role of the P element remains equivocal. Further characterization of similar transformants should determine the precise nature of these integration events. The expression of the P element transposase gene in *A. aegypti* is also being studied. This gene, found on intact P elements, is co-injected with the vector plasmid and its protein product is essential in the mechanism of transposition. An analysis of the processing of this gene in the mosquito embryo may lead to the subsequent use of modified P element constructs which are able to transpose in non-drosophilids.

MOLECULAR CHARACTERIZATION AND PURIFICATION OF THE INSECT PROTHORACICOTROPIC HORMONE IN THE TOBACCO HORNWORM *Manduca sexta*

D.P. Muehleisen, E.J. Katahira, R.S. Gray, and W.E. Bollenbacher

Department of Biology, CB# 3280, Cocker Hall, The University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-3280

The chemistry of the peptide prothoracicotropic hormone (PTTH) is being investigated in *Manduca sexta*. PTTH controls insect molting and metamorphosis by stimulating the prothoracic glands to synthesize ecdysteroids. The neuropeptide exists as two molecular forms,

big PTTH (approx. 28 kDa) and small PTTH (approx. 6.8 kDa). Both forms appear to possess several molecular variants. One variant of big PTTH is being purified by immunoaffinity chromatography using a monoclonal antibody to the peptide.

Progress on the purification and cloning of cDNA for big PTTH will be presented. Data on the physiochemical characteristics of the peptide, such as stokes radius, sedimentation coefficient and its dimeric nature will also be presented.

THE GENE STRUCTURE OF A LYSOZYME FROM *Manduca sexta*

A.B. Mulnix and P.E. Dunn

Department of Entomology, Purdue University, West Lafayette, IN

The hemolymph of naive (non-induced) tobacco hornworm larvae contains a low steady-state concentration of lysozyme. Injection of the larvae with bacteria or bacterial cell wall fragments results in a dramatic increase in the hemolymph lysozyme concentration. RNase protection assays have demonstrated that the increase in the hemolymph concentration is preceded by an increase in the RNAs encoding lysozyme in the fat body. In order to investigate the mechanisms responsible for the lysozyme induction, we have determined the structure of the lysozyme gene. A library of *Manduca sexta* genomic DNA was constructed in the lambda phage vector EMBL3. The library was screened with a probe derived from a previously characterized lysozyme cDNA. Two genomic fragments hybridizing to the probe were selected for study. One of these clones has been characterized and sequenced. The lysozyme coding sequence is divided into three exons. Of the 848 basepairs encoding the lysozyme cDNA, 419 basepairs comprise a 3′ untranslated region. This untranslated region appears to be encoded on a single exon in the gene. The putative promoter region of the gene has been identified.

ISOLATION AND AMINO ACID SEQUENCE OF PHEROMONE BIOSYNTHESIS ACTIVATING NEUROPEPTIDE OF THE SILKWORM, *Bombyx mori*

H. Nagasawa,[1] A. Kitamura,[1] H. Kataoka,[1] T. Inoue,[1] A. Matsumoto,[2] T. Ando,[3] and A. Suzuki[1]

[1] Department of Agricultural Chemistry, The University of Tokyo, Bunkyo-ku, Tokyo 113, Japan
[2] Physics and Chemical Research Institute, Wako, Saitama 305, Japan
[3] Department of Plant Protection, Tokyo University of Agriculture and Technology, Funchu, Tokyo 183, Japan

Most female moths produce sex pheromone(s) in the pheromone glands at the tip of the abdomen. Recently, it was found in several insects that the pheromone production was controlled by a brain peptide termed pheromone-biosynthesis-activating neuropeptide (PBAN). We tried to purify PBAN from head of silkworm, *Bombyx mori*.

PBAN titer was assessed by the amount of sex pheromone, bombykol, produced by the virgin female moth injected with the sample. After many preliminary experiments, the purification scheme for PBAN could be established. Finally, we obtained two distinct PBANs named PBAN-I and -II by a 10-step purification procedure consisting of defatting, 80% aq. ethanol extraction, heat-treatment, acetone precipitation, charcoal column chromatography, gel filtration, ion-exchange chromatography and reverse phase HPLC. PBAN-I and -II were separated from one another at the final step of purification. Both PBANs showed activity at a dose of 0.3-1.0ng.

Complete amino acid sequence of PBAN-I was determined by microsequencing analyses of intact and digested peptides. The results showed that *Bombyx* PBAN-I was a C-terminally

amidated peptide with 33 amino acid residues. Synthetic PBAN-I had chromatographic and biological properties identical with those of the isolated PBAN-I.

ANALYSIS OF SEGMENTATION IN THE LEPIDOPTERAN, *Manduca sexta*

L.M. Nagy, R. Booker, and L.M. Riddiford

Department of Zoology, University of Washington, Seattle

Many insect species from segments with overtly different morphological patterns than *Drosophila*. To understand how these morphological differences are controlled, we have begun to isolate segmentation and homeotic genes from the lepidopteran, *Manduca sexta*. Starting with a *Drosophila antennapedia* homeo box, we used sequence homology to isolate a *Manduca* homeo box. Additional genomic clones with homeo box homology were obtained by rescreening with the *Manduca* homeo box clone. The initial three clones isolated and sequenced appear to be homologues of the *Drosophila antennapedia* and *Bithroax* Complex genes: *Deformed*, *Antennapedia*, and *abdominal*-A. In all three *Manduca* genes the putative amino acid sequence of the homeo box and its immediately flanking regions are identical, or nearly so, to the *Drosophila* genes. Northern blots indicate that the onset of transcription of all three genes occurs before gastrulation. In the 5th instar and pupal stages, *manduca-dfd* RNA is most abundant in the head *Manduca -ant* RNA is absent in the head, most abundant in the thorax, and less abundant in the abdomen, and *Manduca-abd*-A RNA is most abundant in the abdomen. *In situ* hybridization shows that in the 40 hour embryo the anterior boundary of expression of the *Manduca abd*-A gene is the posterior half of the metathorax. These expression patterns directly mimic the expression of the *Drosophila* homoetic genes. (Supported by NIH A1 12459 and Training Grant GM072780 (LMN)).

PhTX

K. Nakanishi, K. Konno, R. Goodnow, R. Bukownik, T. Kallimopoulos, A. Eldefrawi, M. Eldefrawi, and P. Usherwood

Department of Chemistry, Columbia University in the City of New York, New York, NY 10027

The venom of the digger wasp, *Philanthus triangulum* has afforded a new toxin, PhTX-433, which has been characterized as consisting of butyryl-trysoyl-spermine moieties. The toxin has been found to be a non-competitive glutamate receptor antagonist which inhibits the quisqualate sensitive receptor. Over 40 analogues have been synthesized, some of which are more potent than the natural toxin. Currently our efforts are focused on preparing radioactive analogues with photoaffinity groups in order to isolate the receptor. The results of these experiments will be made known in the near future.

CUTICULAR PROTEINS OF *Bombyx mori*: GENE STRUCTURE AND EXPRESSION

H. Nakato, S. Mori, H. Sakurai, S. Izumi, and S. Tomino

Department of Biology, Tokyo Metropolitan University, Tokyo 158, Japan

The urea-soluble fraction of the salt-washed pupal cuticle contains a group of proteins distinct from those found in the larval cuticle. The mRNA sequence for one of the pupal cuticular proteins (PCP) was cloned, and genomic clones for PCP were isolated from a *Bombyx mori* genomic library. The PCP gene is composed of a 147 bp 1st exon and 1047 bp 2nd exon

interspersed by a 5.8 kb intron. The 5′ flanking region of the gene contains a TATA box at position -32 and CAAT box-like sequences at -91 and -104. Northern blot hybridization of the epidermal RNA indicated that the PCP mRNA accumulates in integument at around the period of larval-pupal transformation, though a small and temporal rise of the mRNA level was also noticed at each stage of larval molting. Administration of 20-hydroxyecdysone to the isolated abdomens of the final instar larvae provoked the accumulation of PCP mRNA in integument. The deduced primary structure of PCP revealed the occurrence of two types of unique reiterated sequences; the structure -Thr-Pro-Glu-Val-Ala-Gln-Ala-Arg-Ala-Ala-His- (Motif A) is present in three separate locations, while four consecutive repeats of -Ala-Pro-Ala-His-Gln-Asp/Ser-Trp-Asn- sequence (Motif B) occur in the carboxyl proximal domain. The β-galactosidase/PCP fusion protein containing Motif A and B was selectively adsorbed to the chitin column, suggesting that these repeating structures of PCP might function as chitin-binding domains.

METHYL-BRANCHED ALCOHOLS IN LEPIDOPTERAN PUPAE

D.R. Nelson, and G.J. Blomquist

Biosciences Research Laboratory, USDA-ARS, Fargo, ND, 58105.
Biochemistry Department, University of Nevada-Reno, Reno, NV 89557

Methyl-branched alcohols for C24 to C44 were identified as components of the internal lipids of lepidopteran pupae *Trichoplusia ni*, *Manduca sexta*, and *Diatraea grandosella*. Minor amounts of C14, C16 and C18 alcohols were also identified. The methyl-branched alcohols consisted of 4 homologous series: a monomethyl, two dimethyl and a trimethyl-branched series. The major components of each series had an even number of carbon atoms in the backbone of the molecule. The major components were C40, C42 and C44 dimethyl-branched alcohols with methyl branches on carbons ω-13, and ω-17; next in abundance were dimethyl-branched alcohols with methyl branches on carbons ω-5 and ω-17; then monomethyl branched alcohols with methyl branches on ω-17; and trimethyl-branched alcohols with methyl branches on ω-5, ω-13, and ω-17. In *M. sexta* the major component of each homologous series (with the methylalkane obtained by total reduction in parenthesis) was, respectively: 24-methyltetracontan-1-o1 (17-methyltetracontane), 24,28-dimethyltetracontan-1-o1 (13,17-dimethyltetracontane), 22,34-dimethyloctatriacontan-1-o1 (5,17-dimethyloctatriacontane), and 22,26,34-trimethyloctatriacontan-1-o1 (5,13,17-trimethyloctatriacontane). [1-^{14}C]Propionate was incorporated into the alcohols early in the pupal stages of *M. sexta* reaching a maximum rate at 9-10 days postpupation and falling to zero by 14 days postpupation. Hydrocarbon synthesis had ceased by 8 days postpupation but resumed again the day prior to adult emergence. The very long-chain methyl-branched alcohols have not been found in the surface lipids of either *T. ni* or *M. sexta*. A comparison of the methyl branch positions on the alcohols with those on the hydrocarbons indicated that there was probably not a precursor product relationship between the very long-chain methyl branched alcohols and the very long-chain methyl branched hydrocarbons.

EVIDENCE THAT YOLK GRANULES OF *Blattella germanica* BECOME ACIDIFIED DURING EARLY EMBRYO DEVELOPMENT

J.H. Nordin, and E.L. Beaudoin

Department of Biochemistry, University of Massachusetts, Amherst, MA 01003

Following its uptake into oocytes by endocytosis, vitellin (Vt) concentrates in yolk granules (YGs) and, in *Blattella germanica*, is stored, processed and then utilized for embryo

growth. The mechanism regulating these events is unknown. To ascertain the fate of YGs during this time, yolk of early embryos (0-6 days postovulation) was studied using microscopic and biochemical techniques. Prior to day 4, YG size distribution (15-50 μm) and Vt subunit composition remain constant. At days 4 to 5, yolk protease(s) are expressed coincident with (a) limited processing of 2 of Vt's 3 subunits, (b) a decrease in the average size of the YGs and (c) onset of most embryo growth. We have considered the possibility that a temporally regulated acidification to YGs is involved in the processing/degradative pathway for Vt. To examine this question, yolk was obtained from eggs during early development and incubated with the lipophilic, weak base, acridine orange (AO), which emits an intense orange fluorescence when accumulated in acidic organelles. When yolk was taken at days 4-6 and incubated at pH 6.8, with 50-100 μM AO, most YGs exhibited the orange fluorescence; those from yolk of earlier eggs has only a non-specific green or yellow green fluorescence. The orange vacuoles (3-10 μm) sedimented at 165 g.min confirming their high density. Orange fluorescence was unaffected by selected changes in buffer composition and osmotic strength but was abolished by addition of 50 μM monensin or 0.05% Triton X-100 or by incubation at pH 4.5; conditions which will dissipate trans-membrane pH gradients. The fact that AO accumulation by YGs coincides with the day 4-5 events strongly suggests that their acidification may be a crucial step in the programmed utilization of Vt. Possible roles for acidification include activation of the processing protease(s) and alteration of the integrity/structure of YGs or Vt, facilitating consumption. (Supported by NSF DCB 88-19371.)

THE ALPHA-AMYLASE GENE REGION OF *Drosophila miranda*: A NEWLY EVOLVED DOSAGE COMPENSATED GENE

R.A. Norman, and W.W. Doane

Department of Zoology, Arizona State University, Tempe, AZ 85287-1501

Sex-linked dosage compensation occurs when the level of an X-linked gene product is equivalent in both sexes despite the presence of two X chromosomes in females and a single X in males. The structural genes for alpha-amylase (*Amy*) are autosomal in species of *Drosophila* with the exception of *D. miranda*, where it is located in a newly evolved X-chromosome (X_2) which arose due to an ancestral Y-autosome translocation. *Amy* expression is fully dosage compensated in terms of amylase activity in male larvae of *D. miranda* but apparently not in adults. Sibling species, *D. persimilis* and *D. pseudoobscura*, carry *Amy* autosomally, and provide an unusual opportunity to study the molecular evolutionary basis of a mechanism in insects for gene regulation.

Molecular analysis of the *Amy* region from *D. miranda* shows it contains two Amylase genes (*Amy1*, and *Amy2*) as well as a partial gene (*Amy3*) which contains about 600 base pairs of the 5′ end of the gene. *Amy1* and *Amy2* are complete genes with an inverted orientation relative to one another. Restriction maps of the genes are virtually identical. Individual, cloned *Amy* genes were tested for expression by injection into preblastoderm embryos of an amylase-null strain of *D. melanogaster*. Exogenous *Amy1* produced an active amylase protein whereas *Amy2* did not. Thus, *Amy1* is functional and presumably evolved the necessary regulatory apparatus for dosage compensation in male larvae. Our goal is to characterize that apparatus at the molecular level.

REGULATION OF IMAGINAL WING DISC DEVELOPMENT IN THE INDIAN MEAL MOTH

H. Oberlander, and D.L. Silhacek

USDA/ARS, Insect Attractants, Behavior and Basic Biology Research Laboratory, P.O. Box 14565, Gainesville, FL

The Indian meal moth, *Plodia interpunctella* has been used extensively for studies on the actions of insect hormones and growth regulators. In our laboratory we have studied recently the stimulatory effects of the non-steroidal ecdysteroid mimic RH 5849, (1,2-dibenzoyl-1-tert-butylhydrazine), and the inhibitory effects of 2 chitin synthesis inhibitors, diflubenzuron and teflubenzuron. These agents were administered to intact larvae via their diet, to organ cultures of wing imaginal discs, and to cell lines established in our laboratory from wing discs (IAL-PID2). RH 5849 mimics the effects of 20 hydroxyecdysone on amino sugar uptake in cell lines and on cuticle formation in organ cultures, though ten-fold higher concentrations were required. The presence of extrinsically applied juvenile hormone permits the expression of the ecdysteroidal properties of RH 5849 in larvae in which the formation of cuticularized wings discs was observed only in supernumerary larval instars. The chitin synthesis inhibitors have been examined also. Thus, the Indian meal moth provides an excellent system for studying the actions of developmental hormones and insect growth regulators at the cellular, tissue and organismal levels. These investigations pave the way for molecular investigations of the actions of these compounds.

DEVELOPMENTAL ANALYSIS OF EXPRESSION OF THE FRMFamide NEUROPEPTIDE GENE IN *Drosophila*

M.A. O'Brien, L.E. Schneider, and P.H. Taghert

Department of Anatomy and Neurobiology, Washington University Medical School, St. Louis, MO 63110

We have mapped the pattern of expression of the FMRFamide neuropeptide gene in *Drosophila melanogaster* during development, as a first step in understanding the regulation of this neuropeptide gene. Using an *in situ* hybridization method, we have revealed a discrete pattern of expression which is surprisingly constant during development. There appear to be at least two steady state mRNA levels, the different levels persisting throughout development. The strong hybridization signals, bilaterally paired on the ventral surface of the three thoracic neuromeres and medially paired in the subesophageal ganglion, are observed at all stages of development beginning at about 70% of embryogenesis. These strong hybridization signals probably correspond to neurons that are immunoreactive with different antibodies generated against several regions of the FMRFamide polypeptide precursor (see abstract by Schneider and Taghert). Cellular identities of the numerous weaker hybridization signals are more difficult to determine; in general, the pattern of gene expression determined with *in situ* hybridization correlates well with the combined patterns of immunoreactivity generated with the various antibodies against FMRFamide precursor epitopes. The larval pattern of expression persists through adult development; a small number of new signals appear in the adult stage to complement what is essentially a larval-derived pattern.

Another approach for investigating the regulation of this neuropeptide gene is to create a mutation in the FMRFamide gene, which is located in 46C of the second chromosome. We are currently creating deficiencies in this region using a strain that has a P element inserted in the 46C-D region. The P element insert contains the alcohol dehydrogenase (Adh) gene. Progeny of x-ray mutagenized flies are screened with pentynol to kill Adh-bearing flies. Those individuals that have lost the Adh gene are further screened for loss of adjacent genomic

regions. Mutations in the FMRFamide gene or its regulatory regions should provide novel opportunities to analyze gene regulation and neuropeptide function.

P ELEMENT TRANSPOSASE ACTIVITY IN INSECTS

D.A. O'Brochta,[1,2] S.P. Gomez,[2] A.M. Handler[2]

[1] Center for Agricultural Biotechnology, Department of Entomology, University of Maryland, College Park, MD 20742
[2] Insect Attractants, Behavior and Basic Biology Laboratory, USDA/ARS Gainesville, FL 32608

In an effort to determine if P element based gene vectors can function in non-dropsophilid insects we previously described an *in vivo* P element transposon mobility assay which detects P element excision events from plasmids transiently maintained in insect embryos (O'Brochta and Handler, PNAS 85:6052). The results of this assay indicated that tephritid fruit flies could not support P mobility, and that the ability to support P mobility was a function of relatedness to *Drosophila melanogaster*. However, the excision events detected by this assay were a minority of all excisions. Here we describe a new excision indicator plasmid which allows the unbiased detection and recovery of excision products resulting from *D. melanogaster* and *Chymomyza proncemis*, a related drosophilid, indicate both quantitative and qualitative differences in the activity of transposase in these two species. Evidence for unusual or inefficient transposase mRNA processing in the tephritid *Anastrepha suspensa* is also presented.

PURIFICATION OF THE *Busseola* DIAPAUSE PROTEIN

E.O. Osir

The International Centre for Insect Physiology and Ecology (ICIPE), P.O. Box 30772, Nairobi, Kenya

The *Busseola* diapause protein (BDP) was purified by a combination of density gradient ultracentrifugation, gel permeation and affinity chromatography. It is a high molecular weight protein (M_r 5 x 10^5, pI = 6.1) that is composed of two subunits, 1 (M_r 88,000 ± 4,000) and 11 (M_r 79,000 ± 1,000). The protein contains lipids (2%) and covalently-bound carbohydrates (1%). Amino acids analysis of BDP showed a high tryosine plus phenylalanine content (16 mol%). Labeling experiments using [^{35}S]-methionine showed that *de novo* synthesis of BDP by the fat body occurs only in diapausing larvae. The protein was also shown to bind specifically to 10*R*, 11*S*-epoxybisfarnesyl diazoacetate ([^{3}H]-EBDA), the photoaffinity analog of juvenile hormone 1 (JH 1). We suggest that BDP could serve a storage function by providing the amino acids needed for the synthesis of pupal and adult structures as well as a JH binder during diapause.

JUVENILE HORMONE RECEPTORS IN THE LARVAL EPIDERMIS OF THE TOBACCO HORNWORM

S.R. Palli and L.M. Riddiford

Zoology Department, University of Washington, Seattle, WA 98195

To search for cellular receptors for juvenile hormone (JH) in the epidermis of *Manduca sexta* larvae, we have utilized tritium-labeled photoaffinity analogs of JH, EBDA(JHI), and

EHDA(JHII) and MDK (methoprene). These JHs bind specifically to a 29 kDa protein in the nuclei and to a 38 kDa protein in the cytosol. Binding to both these proteins by EBDA or by EHDA can be prevented with 250-fold excess unlabeled hormone. Binding of MDK to the 29 kDa protein was not prevented by 500-fold excess methoprene at pH 8 but was prevented at pH 9. Two D gels revealed 2, 29 kDa proteins (pI's 5.8 and 6.0) binding MDK; at pH 9.0 methoprene eliminates this binding to the pI 6 form and reduces it to the pI 5.8 form. These high affinity JH binding proteins are not detected either in pupally committed epidermis or in epidermis of larvae allatectomized at the beginning of the final larval instar and therefore devoid of JH, but are present in pupal abdominal epidermis. This reappearance is dependent on the presence of JH during the prepupal ecdysteroid rise. A 29 kDa nuclear protein binds to DNA of two JH-responsive cuticle genes of *M. sexta*. This DNA binding protein and the photoaffinity-labeled 29 kDa nuclear protein showed similar developmental expression. 0.5M KCl extracts of day 1, 5th instar larval nuclei retard the mobility of JH-responsive cuticular gene. These nuclear proteins bind specifically to a 400 bp region in the first intron. We used cDNA clone for the human retinoic acid receptor (RAR) containing a 66 amino acid conserved region present in all hormonal nuclear receptors to search for the JH receptor gene. Southern blot analysis of *M. sexta* genomic DNA under low stringency hybridization conditions showed three distinct EcoR1 fragments. One clone was isolated by screening a *M. sexta* genomic library under the same hybridization conditions. The 1.7 kb *Sal*I fragment which hybridizes to the RAR cDNA was sequenced. Comparison of deduced protein sequence showed that 12 amino acids out of 25 amino acids in the second zinc finger region of the DNA binding region are identical to those in the RAR. Seven of these 12 amino acids are conserved in most known nuclear receptors. (Supported by NSF DCB-88-18876.)

STORAGE AND FOREIGN PROTEIN CLEARING FROM HEMOLYMPH OF PHARATE ADULT SATURNIIDS

M.L. Pan [1] and W.H. Telfer[2]

[1] Department of Zoology University of Tennessee, Knoxville, TN 37966-0801

2 Department of Biology, University of Pennsylvania, Philadelphia, PA 19104-6018.

Over 100 mg of hemolymph storage hexamers disappear during the pupal-adult molt of *Hyalophora cecropia* and *Actias luna*. Selective and non-selective processes must be sorted out to understand how these proteins are utilized. Rocket immunoelectrophoresis showed that in both species the concentration of arylphorin falls slowly at first, and then drops rapidly to an undetectable level at the end of the molt. *H. cecropia* arylphorin injected into *A. luna* disappeared in the same pattern. HSA and ovalbumin injected into *H. cecropia* males disappeared more rapidly than arylphorin during early stages but much more slowly toward the end of the molt. Being relatively protected from earlier protein clearance, arylphorin is thus reserved for use by a selective process at later stages. Vitellogenin is also protected, disappearing after injection into males more slowly than the vertebrate proteins. Hemolymph flavoprotein, a *H. cecropia* storage hexamer that is absent from *A. luna* pupae, disappears in its native species on the same time course as arylphorin. When injected into *A. luna*, however, the timing of its disappearance is very different: in *A. luna* males it disappears more rapidly and completely than in any other protein studied, suggesting precocious activation of a specific clearing mechanism; in females it clears even more slowly than BSA. The results imply: (1) a general mechanism for gradual protein clearing, which can intercept foreign proteins; (2) relative protection of storage hexamers and vitellogenin from this general mechanism; (3) stage-specific mechanisms that selectively clear storage hexamers, but distinguish between them; and (4) species and gender differences in the staging of selective hexamer clearing. Future goals are to identify the cells responsible for clearance, and the molecular traits that protect and target the hexamers. (Supported by NIH grant, GM-329099.)

STIMULATION OF ELECTROLYTE AND FLUID SECRETION BY LEUCOKININ-8 IN ISOLATED MOSQUITO MALPIGHIAN TUBULES; ELECTRON PROBE ANALYSIS AND ELECTROPHYSIOLOGICAL CORRELATES

T. Pannabecker, M. Baustian, T. Hayes, G. Holman, R. Nachman, and K. Beyenbach

Section of Physiology, Cornell University, Ithaca, NY 14853

The insect peptide leucokinin-8 (LK-8) significantly increases the rate of transepithelial (TEP) fluid secretion in a dose-dependent manner with a threshold concentration near 10nM (Life Sci., 1989, 44:1259). LK-8 (16 nM) also significantly depolarizes the TEP voltage (Vt) from +29 to +4 mV. The depolarization is dependent upon peritubular [Cl]. At present we are investigating the effects of LK-8 on TEP electrical resistance and TEP Na, K, and Cl secretion rates to determine the mechanism of action of LK-8. Studies with intracellular microelectrodes and cable analysis show that LK-8 significantly decreases transepithelial resistance (Rt) from 3.8 to 1.1 Kohmcm, with no significant change in fractional resistance of the apical (FRa) and basolateral membranes. The depolarization of Vt arises via a significant depolarization of the apical membrane voltage (Va, +84 mV to +51 mV); the basolateral membrane voltage is not significantly affected. Electron probe analysis (wavelength dispersive spectroscopy) of secreted fluid shows that LK-8 increases the secretion rates of Na (70 to 96 pmol/min, p=.03), K (29 to 73 pmol/min, p=.0001) and Cl (100 to 178 pmol/min, p=.0001). Together, these results show that LK-8 increases TEP NaCl and KCl secretion. The increase in electrolyte secretion involves electroconductive transport across the epithelium. The depolarization of Va, and the Cl dependence of the TEP voltage depolarization suggest that the Cl conductance of the apical membrane increases in response to LK-8. However, since FRa does not decrease, there must be a parallel conductance increase of similar magnitude (ion unknown) at the basolateral membrane. (Supported by NSF grant PCM 8403305 to KB.)

ISOLATION OF PRESUMPTIVE VOLTAGE-SENSITIVE SODIUM CHANNEL GENES FROM INSECTS

L. Payne,[1] L. Pitifer,[1] P. Marsella-Herrick,[1] D. Sun,[1] F. Wong,[2] D.M. Soderlund,[1] and D.C. Knipple[1]

[1] Department of Entomology, New York Agricultural Experiment Station, Cornell University, Geneva, NY 14456
[2] Department of Opthalmology, Duke University Medical Center, Durham, NC 27710.

Voltage-sensitive sodium channels mediate the transient sodium permeability associated with the action potentials of animal nervous systems and are the macromolecular targets of two important classes of neurotoxic insecticides, pyrethroids of this macromolecular target site as the basis for reduced neuronal sensitivity to these compounds, which is one of the most serious problems in insecticide resistance. Because of the central role of sodium channels in insecticide action and resistance, it is of considerable interest to isolate insect genes that code for them. Two genes of *Drosophila melanogaster* that have structural similarity to vertebrate sodium channel genes have been described recently (Salkoff, *et al.*, 1987, Science 237:744-749; Loughney, Kreber and Ganetzky, 1989, Cell 58:1143-1154; Ramaswami and Tanouye, 1989, Proc. Natl. Acad. Sci. USA 86:2079-2082). We have made use of these data to develop several distinct approaches to generate probes for isolating sodium channel genes from other insects. We describe one of these in which we identified regions of high amino acid sequence identity and low condon ambiguity in order to design degenerate oligonucleotide primers that

were used in PCR reactions to amplify short regions of nucleotide sequence (Kamb *et al.*, 1989, Proc. Natl. Acad. Sci. USA 86:4372-4376). This method provided an easy and direct means to generate conspecific probes permitting the isolation of homologues of the *Drosophila* $para^+$ sodium channel gene from several economically important insect species. We also discuss the evidence for the existence of multiple sodium channel genes in *D. melanogaster* in the context of our toxicological and electrophysiological data suggesting that the $para^+$ gene product is a functional sodium channel, which is the principal sit of action of pyrethroid and DDT insecticides.

STRUCTURE AND FUNCTION OF AN IDENTIFIED VENTRAL CORD INTERNEURON (A4I1) IN THE LOCUST

H.-J. Pflüger[1] and M. Burrows[2]

[1] Freie Universität Institut für Neurobiologie, Königin-Luise Str. 28-30, D-1000 Berlin 33, FRG
[2] Zoology Department, University of Cambridge, England

A long ventral cord interneuron has been identified in the locust that extends from the fourth abdominal ganglion (AG4), where its soma lies, to the brain, where its axon terminates in the dorsal deutocerebrum. It receives direct, monosynaptic input in the prothoracic ganglion (PRO) from filiform hairs on the prosternum and windsensitive head hairs (field 1). The longest prosternal hairs, which appear in the first instar, make powerful connections, such that a sensory spike initiates a spike in A4I1. Smaller hairs, which appear later in postembryonic development, make only weak connections so that their sensory spikes produce only epsps of different amplitudes. Spikes of A4I1 that are generated within the PRO, travel anteriorly to the brain and posteriorly through the thoracic ganglia and first three abdominal neuromeres to AG4 where they passively invade the soma. The distribution of synapses upon A4I1 supports the physiological findings (Watson, A.H.D., and Pflüger, H.J., 1989, J. Comp. Neurol. 279:515-527). Ventral branches in the PRO possess mainly input synapses, in the region where the sensory fibers from prosternal and head hairs terminate. Predominantly output synapses are found on the dorsal branches of A4I1 in all thoracic ganglia and in the abdominal neuromeres. Recently we have found direct connections between A4I1 and a motor neurone of a flight steering muscle in the mesothoracic ganglion, which supports our suggestion that the prosternal hairs and A4I1 are involved in flight steering manouevres.

DOES SUPEROXIDE DISMUTASE PLAY A ROLE IN PROTECTION AGAINST PLANT PRO-OXIDANT ALLELOCHEMICALS IN INSECT LARVAE?

C.A. Pritsos, J. Pastore, and R.S. Pardini

Department of Biochemistry, University of Nevada Reno, Reno, NV 89557-0014

Black swallowtail butterfly, *Papilio polyxenes*, southern armyworm, *Spodoptera eridania* and the cabbage looper larvae, *Trichoplusia ni* are naturally resistant to decreasing levels of the dietary pro-oxidants, xanthotoxin and quercetin. Previous work has focused on the role of the polysubstrate monoxygenase enzymes as the insects' primary defense against allelochemcial toxicity. More recently, studies with our three insect model system have suggested that the anti-oxidant enzymes including superoxide dismutase (SOD), catalase, glutathione reductase, glutathione-S-transferase and glutathione-S-transferase-peroxidase activities also contributed to insect larval resistance towards pro-oxidant allelochemicals.

These studies prompted us to test the protective role of SOD towards the pro-oxidant flavonoid quercetin.

Four separate groups of mid fifth instar larvae were fed the following diets: control; quercetin; diethyldithiocarbamate (DETC) an SOD inhibitor; or DETC + quercetin. Data from these experiments show that DETC feeding inhibits SOD in all three insect larvae species. The combination of DETC and quercetin result in synergistic toxicity in *P. polyxenes* and *S. eridania* but the combination had little effect on *T. ni*. These findings indicate that SOD plays a role in the protection of insect larvae from dietary prooxidants. (Supported by USDA competitive grant 88-37153-3475.)

MUSCARINIC RECEPTORS IN LOCUST GANGLIA ARE LINKED TO THE INOSITOL LIPID PATHWAY

S. Qazi and G.G. Lunt

Department of Biochemistry, University of Bath, Bath, England BA2 7AY

We have previously shown that incubation of locust (*Schistocerca gregaria*) supraoesophageal ganglia with muscarinic agonists results in increased turnover of phosphatidyl inositol. We have now examined the effects of such stimulation on the metabolism of the inositol phosphates, $1p_1$, $1P_2$ and $1P_3$. Ganglia were preincubated for varying times in the presence of [^{3}H] inositol and then exposed to muscarinic agonists and/or antagonists. The inositol phosphates were separated by ion exchange chromatography on Dowex-1 resin in the formate form. Incubation of ganglia with [^{3}H] inositol gave high levels of incorporation, particularly into $1P_3$. The enhancement of incorporation by carbachol was seen to be Ca^{++} dependent and Li^+ also enhanced the stimulation. The effects of carbachol were inhibited by atropine.

These data show that muscarinic receptors in locust ganglia are liked to the inositol lipid pathway. Our preliminary experiments indicate that the general characteristics of the insect system are shared with the better characterized vertebrate system, namely cleavage of phosphitdyl inositol by phospholipase C and subsequent production of a variety of inositol phosphates. Whether the insect system shows subtle pharmacological and physiological differences remains to be established.

A NEW CLASS OF TYROSINE-RICH STORAGE PROTEINS PURIFIED FROM THE PUPAE OF TWO WEEVILS, *Sitophilus oryzae* AND *Rhynchophorus palmarum*

Y. Rahbé, B. Delobel, J. Guillaud, and C. Nardon

INRA-INSA, Laboratoire de Biologie Appliquée 406, 20 av. A. Einstein, F. 69621 Villeurbanne cedex, France

Holometabolous insects have developed numerous strategies to meet their developmental needs for aromatic aminoacids used in cuticle incorporation. Among these are a class of aromatic-rich proteins, named arylphorins, and widely studied in Diptera and Lepidoptera. The occurrence of tyrosine-rich storage proteins was investigated in two weevils. Three monomers (two major -44 kDa and 31 kDa and one minor -51 kDa) were purified from nymphs of *Sitophilus oryzae*, and one major monomer (65kDa) from *Rhynchophorus palmarum*. These related proteins belong to a new class of insect storage proteins, tentatively named tyrostaurins, different from either arylphorin or other identified storage proteins. They occur as insoluble granules in the pupal fat body, only extracted by denaturing conditions such as 1% SDS or 8M urea. A convenient tyrosine specific stain was adapted to track them easily during the purification process, which involved preparative SDS-PAGE. The monomers contained no lipids, no phosphate groups and their glycoprotein nature is uncertain, all these features contrasting with the arylphorins. According to their aminoacid compositions, these proteins

may be divided in two subclasses: all of them present three predominant aminoacids amounting to more than 15% of the total aminoacids each (tyrosine, serine and glycine), but the smaller 31 kDa polypeptide from *S. oryzae* exhibits an exceptionally high tyrosine content (more than 27%). The presence of arylphorin in Curculionid hemolymph was also investigated, and the comparative features of the two classes of tyrosine-rich proteins are discussed. As Curculioniadae harbor larval endocytobiotic bacteria involved in a symbiotic supply of aromatic aminoacids, the role of tyrostaurins in the symbiotic relationship is currently under investigation.

CHARACTERIZATION OF HIGH-MOLECULAR WEIGHT PRECURSOR FOR MOSQUITO VITELLOGENIN

A.S. Raikhel and T.S. Dhadialla

Department of Entomology and Program in Cell and Molecular Biology, Michigan State University, East Lansing, MI 48824

In the mosquito, *Aedes aegypti*, the precursor to the major yolk protein, vitellogenin (Vg) is produced by the fat body. Previously, we showed that the mosquito Vg consists of two subunits with Mr=200,000 and 66,000. In this report, we identified a single precursor to both the Vg subunits which exists in the fat body as an intermediate during Vg biosynthesis. The identity of this Vg precursor was confirmed by immunoprecipitation with monoclonal antibodies specific to Vg subunits. The precursor with Mr=250,000 was found in microsomal fractions isolated from vitellogenic fat bodies. With *in vitro* pulse labeling, the 250 kDa precursor could be detected in homogenates of fat bodies from blood-fed mosquitoes only during the first few hours after the initiation of Vg synthesis. Much greater accumulation of the Vg precursor was achieved by *in vitro* stimulation of Vg synthesis in fat bodies cultured with 20-hydroxyecdysone. The 250 kDa precursor was glycosylated and to a much lesser degree also phosphorylated. Treatment of fat bodies with tunicamycin resulted in a precursor which was neither glycosylated not phosphorylated. Its apparent molecular weight (226,000) was similar to that of the Vg primary translation product obtained by cell-free translation of fat body poly(A)$^+$RNA. (Supported by NIH grant AI-24716.)

CONTROL OF PREVITELLOGENIC PROLIFERATION OF RIBOSOMAL RNA IN THE MOSQUITO FAT BODY

A.S. Raikhel[1] and A.O. Lea[2]

[1] Department of Entomology and Program in Cell and Molecular Biology Michigan State University, East Lansing, MI 48824
[2] Department of Entomology, University of Georgia, Athens, GA 30602

During the previtellogenic development of mosquito fat body, the nucleolus, the organelle responsible for producing ribosomes, enlarges three-fold reaching maximal size between 2 and 3 days after eclosion. The granular component of the nucleolus containing ribosomal precursors increases considerably as well. These signs of nucleolar activation correlate with the synthetic rate and accumulation of poly(A)$^-$ RNA (predominantly ribosomal RNA) in the fat body cells. The amount of poly(A)$^-$ RNA in fat body cells increases during the first two days after eclosion and then declines gradually. The rate of RNA synthesis exhibited similar kinetics, but both the rise and decline are sharper than for the accumulation of poly(A)$^-$ RNA. All characteristics of nucleolar activation, its enlargement, accumulation of poly(A)$^-$ RNA and the increased rate of RNA synthesis, are blocked by removal of the corpora allata in newly eclosed adult females but could be restored by either implantation of corpora allata or topical application of juvenile hormone III or its analog, 7-S-methoprene, to

allatectomized females. Thus, previtellogenic activation of fat body nucleoli for ribosomal RNA production is controlled by juvenile hormone from the corpora allata. (Supported by NIH grants AI-24716 and AI-17297.)

TWO DIAPHORASES FROM *Drosophila virilis*: PURIFICATION, BIOCHEMICAL AND IMMUNOCHEMICAL CHARACTERIZATION

K.H. Ralchev, B.C. Dunkov, P.M. Petkov, and T.G. Georgieva

Department of Genetics, Faculty of Biology, University of Sofia, 8 Dragan Tsankov str, Sofia 1421, Bulgaria

Electrophoretic analysis of crude extracts from *Drosophila virilis* revealed the existence of three groups of diaphorases (NAD/P/H:acceptor oxidoreductases) - DIA-1, DIA-2 and DIA-3. A scheme for preparative isolation of DIA-1 and DIA-2 from *D. virilis* adults was developed. It involves different chromatographic techniques. The M.W. of highly purified DIA-1 and DIA-2 were determined as 57 kDa and 66 kDa respectively. Diaphorase-1 reacts with both NADH and NADPH as electron donors. It has a high sensitivity to dicoumarol and it is activated by bovin serum albumin, polyvinylpyrrolidone and Tween 20. These properties of DIA-1 reveal its similarity with mammalian DT-diaphorase and suggest their possible homology. Diaphorase-2 as compared with DIA-1 is only NADH specific and dicoumarol has a slight inhibitory effect on it. Two diaphorases differ by their kinetic properties too. Antibodies against purified diaphorase-2 do not react with diaphorase-1. The results thus obtained indicate the basic difference between two *D. virilis* diaphorases.

Comparative electrophoretic and immunochemical analysis demonstrated a homology between diaphorases of other *Drosophila* species.

MORPHOLOGY AND DISTRIBUTION OF PHEROMONE DETECTING SENSILLAE ON THE ANTENNAE OF *Periplaneta americana*

U.A. Rani

Indian Institute of Chemical Technology, Hyderabad, 500009, (A.P.), India

The various sensilla on the antennae of immature and adult, American cockroach, *Periplaneta americana* was observed with light and scanning electron microscope. The external morphology, density and distribution of pheromone detecting sensillae is identified by comparing the male and female insects. The antenna contains chemosensitive sensilla on every segment. Sensilla basiconica which could be categorized morphologically into four different types, and grooved pegs were found scattered all over the antennal segments. Morphologically there was no difference in the size or shape of each segment. However, on the male antenna there is variation in the density and distribution of sensilla basiconica type III, with long shaft walls, which appear more frequent on all antennal segments. At the basal region they are much more abundant and constitute nearly 50 percent of the sensillum population on the male antennae. The frequency of the s. basiconica type III sensilla on the antenna of the adult male and their presence in smaller numbers in nymphs and adult female insects suggest that these sensilla contain receptors specific for the pheromones of adult females.

SUBUNIT COMPOSITION OF VITELLIN AND VITELLOGENIN IN THE COCKROACH, *Leucophaea maderae*

R.C. Rayne and J.K. Koeppe

Department of Biology, University of North Carolina, Chapel Hill, NC 27599

The predominant form of vitellogenin (VG) in the hemolymph of the cockroach, *Leucophaea maderae*, consists of three subunits designated *A* (112 kDa), *E* (100 kDa), and *C* (52 kDa). Alternative forms of VG contain variants of the *E* subunit, designated *E'* (160 kDa) and *94* (94 kDa), in addition to subunits *A* and *C*. The *E'* subunit is a short-lived precursor to subunit *E*, while *94* is an artifact resulting from enzymatic degradation of *E* in crude extracts of hemolymph.

Western blot analysis using a monoclonal antibody (OV28B) directed toward the *B* (86 kDa) polypeptide of 28S *L. maderae* vitellin (VT) shows that the *E'* and *E* subunits of VG share antigenic determinants with the *D* subunit (94 kDa) in 14 S VT, as well as with the *B* polypeptide itself in both 14S and 28S VT. Thus, subunit *B* in mature VT is the product of sequential modifications of a larger precursor subunit (*E'* to *E* in the hemolymph, *E* to *D*, and *D* to *B* in the oocytes). We hypothesize that the progressive modifications of this subunit provide information essential to successful binding, internalization and compartmentalization of VG/VT by the developing oocyte. Results of recent experiments investigating this hypothesis will be presented.

REPETITIVE ELEMENTS IN THE *Anopheles gambiae* GENOME

R.A. Reiss,[1] R.J. MacIntyre,[1] J. Calvo,[2] and H.H. Hagedorn[3]

[1] Department of Genetics, Cornell University, Ithaca NY
[2] Department of Biochemistry, Cornell University, Ithaca, NY
[3] Department of Entomology and Center for Insect Science, University of Arizona, Tucson, AZ

The biology of mobile genetic elements has been well established in *Drosophila melanogaster*, but little is known about transposable elements in other diptera. We have chosen to search the genome of the malaria vector *Anopheles gambiae* for several reasons. First of all, since it is an extremely important species from a medical standpoint, the natural history of this species has been well characterized. Second, presence of polytene chromosomes in the nurse cell of the ovary make it possible to do *in situ* hybridization with cloned DNA. Two strategies have been employed. The first, using a differential screen of a phage lambda library with labelled DNA from different strains yielded mostly highly repetitive elements. Transposable elements are residents of the in the middle repetitive portion of the DNA. The second strategy is to isolate a gene and mutant alleles, since most spontaneous mutations are caused by insertions. Our model has been the *D. melanogaster* white-eye gene, which was the first mutation to be describe in *D. melangaster*, and the first to be cloned by transposon tagging. A sex linked gene for eye color has been described in *A. gambiae*. Two spontaneous mutants are known for the allele, white-eye and pink-eye. Probing genomic DNA blots of *A. gambiae* DNA with probes from the 3´ end of the *D. melanogaster* white gene reveals homology at lowered stringency. A size difference is associated with the white-eyed and pink-eyed strain. Current efforts are focused on the cloning and characterization of this region.

ENZYMATIC STEPS INVOLVED IN THE 20-HYDROXYECDYSONE REGULATION OF SEX PHEROMONE BIOSYNTHESIS IN THE HOUSEFLY

R.C. Reitz, A.H. Vaz, and G.J. Blomquist

Department of Biochemistry, School of Agriculture, University of Nevada, Reno, NV 89577

(Z)9-Tricosene ((Z)9-23 Hy) is the sex pheromone of the housefly, *Musca domestica*, and is synthesized from oleic acid via elongation to 24:1-CoA followed by conversion to (Z)9-23 Hy. The effects of age, sex and 20-hydroxyecdysone treatments on fatty acyl-CoA elongation, a key reaction sequence in sex pheromone biosynthesis were investigated. Radio-high performance liquid chromatographic analyses of the elongation products of [1-^{14}C]18:1-CoA in the presence of malonyl-CoA and NADPH by microsomes prepared from males of all ages and previtellogenic females showed that all even numbered monounsaturated fatty acids up to 30 carbons were formed. Insects of these ages and sex produce alkenes of 27 carbons and longer. When microsomes prepared from vitellogenic females, which produce (Z)9-23 Hy, were used, there was no elongation of [1-^{14}C]18:1-CoA beyond the 24:1 fatty acyl moiety. Similarly neither [1-^{14}C]22:1-CoA nor [14,14-^{3}H]24:1-CoA were efficiently elongated beyond the 24:1-acyl moiety in mature females. Microsomes from vitellogenic females or males injected with 20-hydroxyecdysone converted [15,16-^{3}H]24:1 CoA to (Z)9-23 Hy, whereas microsomes from males and previtellogenic females did not. NADPH was required for the conversion of 24:1-Coa to (Z)9-23 Hy. These data show that 20-hydroxyecdysone regulates sex pheromone production by altering elongation activity such that (Z)9-23 Hy becomes a major product.

MITOCHONDRIAL DNA RESTRICTION FRAGMENT VARIATION AMONG FOUR GEOGRAPHIC ISOLATES OF THE BOLL WEEVIL, *Anthonomus grandis*

R.L. Roehrdanz

Insect Genetics and Cellular Biology, USDA, Biosciences Research Lab, Fargo, ND

The boll weevil as a cotton pest apparently originated in Mexico and spread rapidly across the southeastern cotton belt at the turn of the century. Several geographic subpopulations have been recognized that differ in host plant preference and possibly morphology. Restriction fragment patterns have been compared among four different laboratory colonies using sixteen restriction endonucleases. The colonies include: 1) the Ebony mutant line, a representative of southeastern cotton weevil; 2) Mexican weevils collected on the native plant *Hampea nutricia*; 3) thurberia weevils collected on the wild cotton *Gossypium thurberi* in the San Molino mountains of Arizona; 4) weevils collected on cotton in El Salvador. The boll weevil mtDNA is about 19 kb. Ten of the enzymes produced polymorphic fragment patterns between two or more pairs of lines. Comparisons of the fragments common to pairs of lines suggests that the southeastern, Mexican and thurberia weevils are about equally dissimilar (82-86% shared fragments). The El Salvador weevil appears to be more closely related to the thurberia weevil (88% shared fragments) than to either of the other two (76-79% shared fragments). The existence of mtDNA variation among these lines suggests that this approach will be suitable for a more detailed analysis of the origin and dispersal of host plant and geographic variants of the boll weevil. A restriction site map for the Ebony strain has been prepared and *Eco*Rl fragments comprising the entire mt genome have been cloned. Preliminary data has revealed two small reiterated sequences that appear to be located int he A+T rich region.

THE VITELLOGENIN GENES OF THE MALARIA VECTOR *Anopheles gambiae*

P. Romans

Malaria Section, LPD/NIAID, National Institutes of Health, Bethesda, MD 20892

Regulatory sequences from genes that are transcriptionally activated to high levels after a blood meal in *Anopheles gambiae*, the major vector of falciparum malaria in sub-Saharan Africa, have been identified. They are of interest for studies of reproduction and for expression of synthetic malaria refractoriness genes. Clones encoding vitellogenin genes were purified from *A. gambiae* genomic library in EMBL 3 by differential screening with first strand cDNA made from non blood-fed adult female mosquitoes and their blood-fed sisters 24 hours after the blood meal. Seven of approximately 20 clones purified have been studied in detail. The gene containing regions were identified by their hybridization on Southern blots of suitable restriction digests with coding region probes from the *Aedes aegypti* vitellogenin gene Al (Hamblin et al., 1987, Mem. Inst. Oswaldo Cruz 82, Suppl. III:109-114). Several of the clones contain portions of two genes an suitably reiterated restriction sites. This and evidence from Southern blots of DNAs prepared from individual mosquitoes indicate that three of the four *A. gambiae* vitellogenin genes are tandemly arranged. Nucleotide sequence has been determined for 12.2 kb of a clone containing one and a part genes a the apparent left end of the array. The gene coding region spans 6.3 kb and the intergenic region including the 3´ untranslated region of the distal gene and the 5´ region of the proximal gene is 3.0 kb. Sequence of the coding regions is virtually identical. The intergenic sequence of these two genes and additional sequence of five other promoter regions show very few base changes between genes. Analysis of the putative regulatory and coding region sequences shows that they contain consensus eukaryotic TATA boxes, duplicate mRNA cap sites, translational initiation sites and signal sequences. Tandem arrangement of vitellogenin genes has not been observed previously. The isolation of only one type of gene suggests that all vitellogenin subunits are processed from the same primary transcript.

VITELLOGENESIS IN THE HARD TICK *Dermacentor variabilis*

R. Rosell,[1] and L.B. Coons[2]

[1] Department of Entomology University of Arizona, Tucson, AZ
[2] Department of Biology, Memphis State University, Memphis, TN

Vitellin proteins from the eggs of the hard tick, *Dermacentor variabilis* were purified. The vitellins were hemoglycolipoproteins as detected by selective staining of polyacrylamide gels, carbohydrate analysis and lipid analysis. Polyclonal antibodies directed against the vitellin were used in immunolabelling at the ultrastructural level to detect the presence of vitellin and the precursor protein, vitellogenin, in the organs of feeding and ovipositing *D. variabilis* females. Vitellogenin was localized in the fat body trophocytes and in the midgut vitellogenic cells of feeding and ovipositing females. Immunoreactive vitellin was also localized in the Golgi bodies and developing yolk bodies of oocytes during early feeding. Vitellogenin uptake was detected in coated pits and coated vesicles at the apical plasma membranes of developing oocytes during feeding and oviposition. In this tick, there appear to be three different organ sources of vitellogenin and the initiation of vitellogenin production in all three organs coincides with the initiation of feeding.

LYSOZYME AND BACTERICIDAL FACTORS IN THE MIDGUT OF *Manduca sexta* DURING METAMORPHOSIS

V.W. Russell and P.E. Dunn

Department of Entomology, Purdue University, West Lafayette, IN 47907

Insects respond to bacterial infection by synthesizing several antibacterial proteins, one of which is lysozyme, a bacteriolytic enzyme that hydrolyzes the glycosidic bonds of peptidoglycan found in bacterial cell walls. A preliminary study of a variety of tissues showed that the midgut contained mRNA specific for lysozyme and that lysozyme activity was present in midgut tissue homogenates during the early fifth instar in both bacterially challenged and naive insects. However, no lysozyme activity was detected in the gut luminal contents. A study of larval midgut at later stages during the instar showed increasing levels of lysozyme activity in tissue homogenates starting at the "wandering period" just prior to apolysis. No lysozyme activity was detected in the midgut lumen until the pupal molt. Histological studies using immunolocalization with colloidal gold conjugated antibodies failed to detect lysozyme in the midgut epithelium of early fifth instar larvae, but the protein was readily localized in the regenerative cells which replace the larval midgut epithelium and give rise to the pupal midgut epithelium. These regenerative cells first become obvious at the "wandering stage", arising from inconspicuous stem cells. By apolysis, they form a continuous epithelium basal to the degenerating larval midgut epithelium. Lysozyme appears in large, single, apical vacuoles characterized by long, irregular microvillae and is concentrated in small dense aggregates. Golgi like vesicles are found in close proximity to the apical vacuoles and possibly give rise to these structures. When the larval epithelium is sloughed off at pupation, the apical vacuoles release their contents into the gut lumen. At this time, high levels of lysozyme and bactericidal activity were detected in the midgut lumen. Lysozyme and bactericidin secretion into the lumen during such a time of gut restructuring might serve a prophylactic role in protecting against bacterial invasion.

NUCLEAR PROTEINS THAT INTERACT WITH THE 5´ FLANKING REGIONS OF STORAGE PROTEIN GENES OF *Bombyx mori*

H. Sakurai, S. Izumi, and S. Tomino

Department of Biology, Tokyo Metropolitan University, Tokyo 158, Japan

Two forms of storage proteins termed as SP 1 and SP 2, respectively are synthesized in the fat body of the actively feeding larvae of *Bombyx mori*. To identify the *trans*-acting factors which regulate transcription of these storage protein genes, we analyzed nuclear proteins that interact with the 5´ flanking sequences of each structural gene. When the nuclear extract of the larval fat body was incubated with the specific DNA fragment spanning from -96 to +46 of the SP 1 gene, two specific DNA-protein complexes were recognized by gel retardation analysis. The extract of the cultured *B. mori* cells, but not those of the silk gland and HeLa cells, also formed these complexes, though much reduced in quantity. Competition experiments and DNase I footprinting analysis concluded that the fat body nuclear proteins interact with the -72 TGATAATG -65 sequence of the SP 1 gene. This sequence is also present in the -94 to -87 region of the SP 2 gene. A probe DNA constructed from the SP 2 gene (nt position -138 to +43) formed three complexes with the nuclear extract. Two of these complexes exhibited electrophoretic mobilities identical to those of complexes from the SP 1 gene. The third complex was specific to the SP 2 gene formed by interaction of nuclear protein(s) with the DNA sequence between -121 and -110 of the gene. These results strongly suggest that the TGATAATG motif is one of the crucial *cis*-acting elements for the co-ordinate or tissue-specific expression of the SP 1 and SP 2 genes.

LIPID REGULATION IN STARVING AND FEEDING FIFTH INSTAR LARVAE OF *Manduca sexta*

S.J. Sanders, B.R. Patterson, K. Tsuchida, and M.A. Wells

Department of Biochemistry, Biosciences West, University of Arizona, Tucson, AZ 85721

Earlier studies of Tsuchida and Wells (1988, Insect Biochem., 18:263) and Tsuchida et al. (1987, Insect Biochem., 17:1139) have shown there is an increase in the lipid content of lipophorin upon the cessation of feeding, whether during the intermolt period or upon pupation. There appeared to be a correlation between the ecdysteroid titers and the lipophorin density, which suggests that changes in ecdysteroid titers may trigger the lipid release responsible for the change in lipophorin composition. In the present studies the effect of starvation on the lipid content in the lipohorin of fifth instar, D-1.5 larvae show that there is also an increase in the lipid content in lipophorin after 24 hours of starvation. Preliminary studies of ecdysteroid titer in starved larvae showed that ecdysteroid was not the cause of increased lipid content in the hemolymph. Experiments are being conducted, using radiolabeled triolein, to study the effect of starvation on the lipid uptake and content in the midgut, in the midgut tissue, in the hemolymph and in the fat body, in order to determine the source of the lipid released during the lipophorin transformation.

A phenomenon noted during the preparation of lipophorin from feeding, 5th instar larvae by 16 hours of ultracentrifugation was the presence of two density bands of lipophorin. Further investigations, using immunodiffusion techniques, have shown the two density bands to be present in individual animals. More experiments are being conducted on carefully staged, fifth instar larvae to determine when the two bands appear, the lip composition of the bands, and the distribution of radiolabeled lip between the bands. (Supported by NIH grant HL-39116.)

20-HYDROXYECDYSONE INDUCED PHOSPHORYLATION OF ACID PHOSPHATASE IN THE FAT BODY OF A LEPIDOPTERAN LARVAE, *Mamestra brassicae*

M. Sass

Department of General Zoology, Eotvos University, Budapest, Hungary

During the feeding period of the last larval stage 20-hydroxyecdysone treatment induces the appearance of the auto- and heterophagic vacuoles and the increase of the activity of lysosomal acid phosphatase in the cytoplasm of fat body cells. In contrast, during the wandering period, when the number and size of lysosomal elements increase dramatically, the activity of acid phosphatase sharply decreases. At the same time hormone-induced phosphorylation of certain fat body proteins has been observed. After fractionation of fat body cells one of the phosphorylated proteins was localized in the fraction in the fraction containing the heterophagic protein storage granules. The protein, which has acid phosphatase activity has been isolated from the whole body of 400 insects and a rabbit antibody has been developed against it. It has been established that this antibody shows cross-reaction in a western-blot with the phosphorylated electrophoretic band which was found to have enzymatic activity during the feeding period. The presence of the enzymatically inactive acid phosphatase in the protein storage granules was proved by immunocytochemical methods. The observed phosphorylation of the lysosomal enzyme may block its activity inside the storage granules and in this way the accumulated proteins are preserved in intact form until the middle of the pupal stage, when they are used up in the differentiation of imaginal cells.

DETECTION OF A POLYMORPHIC BAND COSEGREGATING IN ISOGENEIC POPULATIONS OF *Heliothis virescens* WITH THE PERMETHRIN-RESISTANT PHENOTYPE

K.C. Saucier and T.M. Brown

Department of Entomology, Clemson University,
Clemson, SC 29634-0365

Preliminary Southern hybridizations with numerous single copy DNA probes developed from resistant *Heliothis virescens* colony have resulted in detection of a polymorphic pattern between isogenic strains of *H. virescens* that is resistant status dependent. The clone designated 4B7 recognizes a *Bam*H1 restriction polymorphism within *H. virescens* (lower bands), 4B7 detects a 4.5 kb band in individuals from the permethrin-resistant colony RR (Fig. 1). The isogenic, permethrin-susceptible colony, WOF, does not carry the genomic material corresponding to the 4.5 kb band in resistant individuals. (Overexposure of the autoradiogram for 1 week did not result in a 4.5 kb band in the susceptible individuals.) These results, although exciting, are being examined further on RR to WOF crosses, as well as on other non-isogenic laboratory strains of *H. virescens* to determine the cosegregation of the resistant phenotype with the 4B7/BamH1 4.5 kb band.

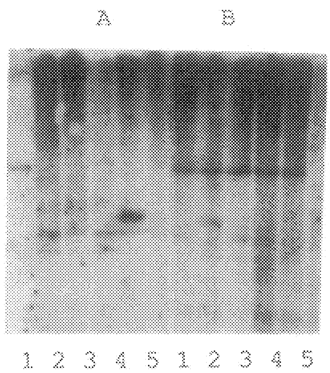


Figure 1: Panel A; Lane 1 Marker, Lane 2-6 WOF DNA digested with *Bam*H1

Panel B: Lane 1-5 RR DNA digested with *Bam*H1

All DNAs were hybridized to ^{32}P labelled clone #4B7 overnight at 42°C, washed and exposed for 18 hrs to Xray film.

SIGNAL TRANSDUCTIONS AND CONTROL OF TICK SALIVARY GLAND FUNCTION

J.R. Sauer, J.L. McSwain, A.E. Qurehis, J.S. Tucker, and R.C. Essenberg

Departments of Entomology and Biochemistry, Oklahoma State University,
Stillwater, OK 74078

Ixodid tick salivary glands are richly innervated and, based upon pharmacological studies, dopamine is a neurotransmitter at the neuroeffector junction controlling fluid secretion. Dopamine stimulates fluid secretion by isolated salivary glands when Ca^{2+} is present in the external medium and dopamine causes salivation when injected into whole ticks. Dopamine stimulated adenylate cyclase increases the level of cAMP which activates a cAMP-dependent protein kinase resulting in phosphorylated proteins. One possible function of phosphoprotein is related to an ability of cAMP and the catalytic subunit of cAMP-dependent protein kinase to affect $Na,^{+}$ K^{+}-ATPase activity in a plasma membrane fraction of the salivary glands.

An additional signal transduction pathway in the salivary glands relates to turnover of

inositol phospholipids. A heat and trypsin-sensitive factor from tick brain (but not dopamine) stimulates formation of inositol phosphates in the salivary glands. Inositol triphosphate (IP_3) stimulates release of Ca^{2+} from the microsomal fraction and Ca^{2+} and diacylglycerol dependent protein kinase C activity has been isolated. Micromolar Ca^{2+} activates a ouabain-insensitive, vanadate-sensitive ATPase in the plasma membrane fraction of the salivary glands.

GENES AND mRNAS OF ADIPOKINETIC HORMONE I AND II

M.H. Schaffer and B.E. Noyes

University of Texas Southwestern Medical Center, Dallas, TX 75235-9070

Nearly full length cDNA copies of the mRNAs of adipokinetic hormones I and II have been isolated from a *Schistocera nitans* corpora cardiaca library and multiple independent isolates have been sequenced. The messages are both about 550 nucleotides in length, and priming experiments demonstrate that the non-poly A sequences are 385 and 380 nucleotides. The relative abundance of the two messages as judged by frequency of recombinants in the library or strength of hybridization signal on RNA blots parallels the levels of two peptides in the corpora cardiaca suggesting that peptide levels are significantly regulated at the nucleic acid level. The messages code for small protein precursors (63 and 61 amino acids) which each begin with a signal sequence followed by the AKH sequence, a gly and double basic processing site, and C-terminal peptide of unknown function. The messages show 64% nucleotide identity which is confined to the coding region and predict precursors which are 56% identical. DNA blotting suggests that AKH I is encoded by a single gene. AKH II specific probes give a more complex pattern suggesting a second gene or a single gene with a large intron. DNA blots of corpora cardiaca brain, and testes show that the high level of AKH I found in the corpora cardiaca is not the result of extensive gene amplification. A genomic recombinant from *Manduca sexta* suggests that M-AKH has a similar mRNA and protein precursor (J.Y. Bradfield, and L. Keeley, 1989, J. Biol. Chem. 264:12791-12793.) (Supported by the Meadows Foundation, the Biological Humanics Foundation, and the Southwestern Medical Foundation.)

DIRECT MEASUREMENTS OF NUMBER, SIZE AND JUVENILE HORMONE BIOSYNTHESIS OF DISSOCIATED CORPUS ALLATUM CELLS

C. Schal, A.-S. Chiang, and M. Gadot

Department of Entomology, Cook College, Rutgers University, New Brunswick, NJ 08903

The corpus allatum of the oviparous German cockroach, *Blattella germanica*, shows cyclic changes in both size and juvenile hormone-III synthesis during the gonotorphic cycle of the female. Changes in number and size of corpus allatum cells were directly measured in cell suspensions of enzymatically dissociated fresh glands. Cell number was verified by total cell counts in whole-mounts of fresh corpora allata. The average cell diameter increases significantly during oocyte maturation, while cell number is relatively stable throughout this period, suggesting that the activation of the corpus allatum during its maturation is mainly achieved through an increase in volume, rather than in cell number. Dissociated cells of active corpora allata incorporated [methyl-^{3}H]methionine into juvenile hormone-III at similar rates to intact glands during short-term incubations in modified TC199. This is the first demonstration of the competence of single corpus allatum cells to synthesize juvenile hormone *in vitro*.

A HEMOLYMPH PROTEIN SPECIFIC TO LARVAL HONEY BEES

J.O. Schmidt, P. Rayces, S.C. Thoenes, J.K. Kawooya, and J. Law

Carl Hayden Bee Research Center, ARS-USDA, 2000 E. Allen Road, Tucson, AZ
Department of Biochemistry, University of Arizona, Tucson, AZ

Honey bee larval hemolymph contains a 640 kDa protein as analyzed by gel filtration and by electrophoresis under native conditions. On SDS-PAGE slab gels, the protein has an apparent molecular weight of 105 kd, thus suggesting it to be a hexamer. Analysis of both adult and larval hemolymph indicates the protein to be confined to the larval stage. The protein was purified to homogeneity by two different procedures. The first involved gradient ultracentrifugation in KBr solution at 50,000 rpm in an VTi50 to remove lipophorin and another protein called "very high density lipoprotein", gel filtration on Bio-Gel A-1.5, affinity chromatography on CM Bio-Gel A, and hydroxylapatite chromatography on Bio-Gel HT. The protein was unstable during these procedures and the yields were low. The second, and much simpler, purification consisted of gradient ultracentrifugation followed precipitation with 20% ammonium sulfate. The protein is rich in glutamate and aspartate, but poor in methionine and phenylalanine. The protein contains 1.3 percent carbohydrate of which mannose predominates. The protein is not present in the hemolymph of *Manduca sexta* or even the closely related carpenter bee species *Xylocopa californica*. The developmental significance of this protein in honey bees is being investigated with antibody probes to elucidate its times and sites of syntheses and uptake and this possible function in the social biology of honey bees.

MOLECULAR CHARACTERIZATION AND CELLULAR ANALYSIS OF THE FMRFamide GENE IN *Drosophila melanogaster*

L.E. Schneider and P.H. Taghert

Department of Anatomy and Neurobiology, Washington University Medical School, St. Louis, MO 63110

We are interested in the mechanisms that underlie neuropeptide gene regulation. To this end we are studying the FMRFamide gene in *Drosophila melanogaster* which encodes a set of thirteen FMRFamide-related peptides (Schneider and Taghert, 1988, PNAS 85:1993; Nambu, et al., 1988, Neuron 1:55). We have characterized the structure of the gene; it is composed of two exons separated by an intron of ~2.5 kb. The second exon encodes the entire polyprotein precursor. A single transcript of ~1.7 kb is detectable throughout all post-embryonic stages. The promoter region of the FMRFamide gene was being compared to its homologue in *Drosophila virilis* to identify possible regulatory sequence elements. To map the expression of this gene we have generated antisera against the peptide sequence Phe-Met-Arg-Phe-amide and against different small peptide epitopes of the FMRFamide precursor. A total of ~60 larval neurons are labelled by the antisera against Phe-Met-Arg-Phe-amide and a subset of these are also labelled by the precursor antisera. In addition, we observed *in situ* hybridization signals in most of the same neuronal regions (see paper by O'Brien et al., this volume) Based on axonal projections of the immunoreactive neurons, the cells that express the FMRFamide gene are apparently of different functional types. Some have processes that project to known neurosecretory sites and probably release peptides into the hemolymph, while others have axons that are confined to the central nervous system and probably function as interneurons. To determin how the FMRFamide gene is expressed in different types of neurons, we are using P element transformations to analyse the regulatory regions of the gene.

PREPARATION OF MONOCLONAL ANTIBODIES FOR NEUROENDOCRINE STUDIES IN THE COLORADO POTATO BEETLE

H. Schooneveld and H.M. Smid

Department of Entomology, Agricultural University, Binnenhaven 7, 6709 PD Wageningen, The Netherlands

This poster shows the procedures for the generation of monoclonal antibodies (MACs) recognizing specific neurons and some other cells in the nervous and endocrine system. The MACs were obtained by injecting mice with crude homogenates of nervous and neuroendocrine tissue. The following factors influence the degree of success. (1) Pretreatment of the beetles as a donor of unspecified antigen mixtures; (2) Dissection of nervous or endocrine tissues and preparation of the immunogen; (3) Strategy for immunization of mice; (r) Assay techniques for testing antibody production by hybridomas and harvesting of MACs.

A surprisingly high proportion of hybridomas produced antibody that recognized specific neurons and other cells of the CNS/NES, following immunocytochemical procedures on light- or electron microscope sections. The following major cell types were revealed:

1. Putative peptidergic neurons and their terminals in diverse locations.
2. Non-specific central or peripheral neurons or their extensions
3. Intrinsic glandular cells in the corpus cardiacum
4. Specific neuroglia cells in restricted brain areas.
5. Various nonneural cells associated with the brain, such as fat cells, oenocytes, haemocytes, and certain epithelial cells in alimentary and reproductive organs.

The panel of 18 MACs recovered so far seems to be useful for mapping of peptidergic centers; the study of neuron-neuroendocrine interactions or innervations; the delineation of axon pathways; the immuno-aided separation of immunoreactive antigens for structure and function elucidation. (Schooneveld, H., Smid, H.M., Boonekamp, P.M. and van Haeften, T., 1989, Cell Tissue Res. 257:29-39)

THE INSECTICIDAL AND NEUROPHYSIOLOGICAL EFFECTS OF THE ACETYLCHOLINE TRANSPORT INHIBITOR, VESAMICOL (AH5183)

M.E. Schroeder,[1] S.D. Buckingham,[2] S.C.R. Lummis,[2] and D.B. Sattelle[2]

[1] E.I. Dupont de Nemours & Co., Inc., Wilmington, DE.
[2] University of Cambridge, Cambridge, U.K.

The toxicological properties of the potent vertebrate neuromuscular blocking agent, vesamicol (AH 5183), were investigated in insects. Vesamicol is not toxic to adult houseflies by topical application. However, insecticidal activity is significantly improved if the flies are pretreated with the oxidative enzyme inhibitor, piperonyl butoxide, or if vesamicol is applied with solvents which promote penetration across the insect cuticle. Also, vesamicol acts as a synergist when applied with the commercial insecticides Pyrin® and allethrin. Mode of action studies on insect nerve tissue show that veasmicol produces a frequency dependent block of nerve impulse transmission at a cholinergic synapse in the cockroach central nervous system at submicromolar concentrations. The experimental conditions required to produce the block are consistent with an effect of vesamicol on the process of neurotransmitter replenishment. Vesamicol also reduces the sensitivity of postsynaptic receptors to acetylcholine and other ligands which interact with acetylcholine receptors in insects. These studies show that vesamicol has intrinsic insecticidal activity which is manifested only under conditions where the insect's

metabolic ability is compromised or penetration across the cuticle is enhanced. Mode of action studies on insect nerve tissue indicate that vesamicol affects both the process of acetylcholine replenishment and the sensitivity of postsynaptic receptors to acetylcholine in the insect central nervous system. The ability of vesamicol to increase the insecticidal activity of Pydrin® and allethrin raises the interesting possibility that compounds like vesamicol could be used as synergists to decrease the amount of insecticide needed to achieve an acceptable level of insect control with obvious benefits to the environment.

TRANSFORMER, TRANSFORMER-2^{TS2} AND *INTERSEX* GENES INFLUENCE ADULT ECDYSTEROID CONTENT IN *Drosophila melanogaster*

M.B. Schwartz,[1] T.J. Kelly,[2] and R.B. Imberski[3]

[1] Department of Zoology University of Vermont, Burlington, VT
[2] USDA, Insect Reproduction Laboratory, Beltsville, MD
[3] Department of Zoology, University of Maryland, College Park, MD

A small set of regulatory genes determines sexual differentiation in *Drosophila melanogaster.* At the molecular level, expression of the yolk polypeptides (YPs) is indicative of tissue and sex-specific female differentiation due to these genes. We have examined *transformer, transformer-2*^{ts2} and *intersex* for a related adult female-specific characteristic, ecdysteroid production. The ovary is an established source of ecdysteroid in the adult. Both *transformer* and *transformer-2*^{ts2} mutations produce pseudomales, chromosomal females morphologically indistinguishable from males. Determination of ecdysteroid levels by radioimmunoassay (RIA) indicates that these flies have male levels of ecdysteroid. RIA of *intersex* females, morphologically a mix of male and female traits, indicates low levels of ecdysteroid in whole body extracts. RIA of these ovaries/gonads indicates low levels of ecdysteroid production. Previously published works show a correlation between the presence of ovary-like gonads and YPs, through our work this extends to ecdysteroid. Thus, the functional state of the gonad allows expression of a sex-appropriate hormone and YP production. Ecdysteroid, in this example, is analogous to vertebrate sex hormones.

SCREENING AND SEQUENCING OF BAG AND TAG cDNA LIBRARIES FOR STRUCTURAL PROTEINS OF *Tenebrio* SPERMATOPHORE

M.B. Schwartz, G. Paesen, and G.M. Happ

Zoology Department, Marsh Life Science Bldg., University of Vermont, Burlington, VT 05405

In male *Tenebrio molitor* (mealworm) two types of accessory sex glands are described: bean-shaped glands (BAGs) and tubular glands (TAGs). Monoclonal antibodies against BAG and TAG proteins allowed localization of specific proteins within BAG, TAG and spermatophore. The antibodies are used to screen cDNA libraries, prepared from BAG or TAG mRNA of six day old adult males, using λgt11 (Promega) and λZAP11 (Stratagene) expression vectors. Use of one monoclonal, designated PL 21.1, against a 23 kDa structural protein of spermatophore produced by BAG, has allowed the isolation of 13 λgt11 clones producing fusion proteins immunoreactive with PL 21.1 Several of the λgt11 clones are being subcloned into Bluescript (Stratagene) for sequencing.

GENES THAT CONTROL PATTERN FORMATION IN *Drosophila*

M.P. Scott, S. Hayashi, J.E. Hooper, and G.M. Winslow

Department of Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, CO 80309

The development of the fruit fly *Drosophila melanogaster* is controlled by a network of regulatory genes. Mutations in segmentation genes alter the number or pattern of body segments in the embryo. Mutations in homeotic genes lead to the development of one part of the body in the pattern of another. Two central problems are to discover how temporal and spatial control of regulatory gene expression is accomplished and to learn how the products of the regulatory genes control the formation of morphology. Many of the regulatory genes appear to encode the transcription factors on the basis that they contain a "homeobox", a 183 base pair sequence that encodes an evolutionarily conserved 61 amino acid protein sequence (the homeodomain) that is a sequence-specific DNA-binding domain. Using *in vitro* DNA binding studies and a cultured cell transfection system we have investigated the transcription-regulating capabilities of some of the segmentation and homeotic gene protein products, and have characterized cis-acting DNA sequences upon which the proteins act to control transcription. Not all of the segmentation genes and homeotic genes contain homeoboxes. The segmentation gene *patched* (ptc) controls pattern formation in part of each body segment. The *patched* gene is expressed in a complex and dynamic pattern of transverse stripes. Sequence analysis of the gene has revealed that it encodes a product that may be a transmembrane protein. Therefore this gene may function in cell-cell communication rather than in transcriptional regulation.

THE ROLE OF EXTERNAL GONADAL SHEATH IN SEX DIFFERENTIATION OF GERM CELLS IN *Bombyx mori*

E.A. Shchegelskaya and V.V. Klimenko

Institute of Ecology and Genetics, Lesnaya 20, Kishinev 277018, USSR

To study the role of the gonadal sheath in sex differentiation of germ cells in *Bombyx mori*, we developed the following experimental system: removal of the external sheath of donor's gonad, implantation of the gonad into recipient's body at the larval instar, and cytological analysis of implanted gonad. The testes were implanted into females and males at the second instar when germ cells were at premeiotic stages. The implants were removed from the recipients at fifth instar and studies cytologically. In all testes, matured without external sheath in females, phenotypically female sex cells were observed. They can be easily recognized by their big size, differentiation into oocyte-trophocyte complexes, and specific chromatic structure. We found 30-300 phenotypically female cells in every testicular lobe. They developed both as single trophocytes and cysts containing 2-16 cells. In 22.6% of testes matures without external sheath in males, 10-60 phenotypically female cells were observed in each testicular lobe. Only male sext cells were found in undamaged testes implanted into females and males. We suggest, that the external testicular sheath functions like the blood-testis barrier for feminizing factors which appear to exist in all females and in some males at second instar. The presence of gonadal sheath plays an important role for normal differentiation of male sex cells. Phenotypic sex reversal observed in the male germ line of *Bombyx mori* can be considered as an approach to study the mechanism of germ line differentiation using molecular biological techniques.

EVIDENCE FOR A JUVENILE HORMONE RECEPTOR IN TISSUES OF *Drosophila melanogaster*

L. Shemshedini and T.G. Wilson

Department of Zoology, University of Vermont, Burlington, VT 05405

Using [^{3}H] JH III as well as the Jh III photoaffinity analy epoxy farnesyl diazoacetate (EFDA), we have identified from cytosol of several tissues in *Drosophila* a major protein that possesses characteristics expected of a Jh receptor. Based on SDS gel electrophoresis the protein has a MMW of 94,000 and is found in several pupative JH target tissues, including larval fat body, male accessory glands, and larval epidermis. It is not found in thoracic muscle, gut or (surprisingly) ovary. A similar protein is also found in nuclei from larval fat body. Preparations from either larval fat body of male accessory glands have the following characteristic: 1) binding is saturable by JH III, 2) binding is specific for Jh III, 3) JH III binds with high affinity (K_D-5nM), *Methoprene-tolerant* (*Met*), and 5) stimulation of RNA and protein synthesis by JH III in male accessory glands cultured *in vitro* is 2-3 fold lower in *Met* flies than in wild-type.

We believe this protein mediated the action of JH III in *Drosophila melanogaster.*

NEW CLASSIFICATION OF BOMBYCID AND SATURNIID SILKMOTHS BASED ON THE STRUCTURES OF ARYLPHORINS

T. Shimada,[1] J. Kobayahi,[2] M. Nagata,[3] H. Maekawa,[1] and S. Izumi[4]

[1] National Institute of Health, Tokyo 141, Japan
[2] National Institute of Sericultural and Entomological Science, Ibaraki 305, Japan
[3] Department of Agrobiology, Tokyo University, Tokyo 113, Japan
[4] Department of Biology, Tokyo Metropolitan University, Tokyo 158, Japan

We investigated the immunological crossreactivity of arylphorins among silkmoth species by Oudin's and Ouchterlony's immunodiffusion tests to re-examine morphological classification of the silkmoths. Material insects are 1) *Bombyx mori*, 2) *Bombyx mandarina*, 3) *Prismosticta hyalinata* 4) *Oberthueria falcigera*, 5) *Pseudandraca gracilis*, 6) *Samia cynthia ricini*, 7) *Samia cynthia pryeri*, 8) *Antheraea yamamai*, 9) *Antheraea pernyi*, 10) *Dictyoploca japonica*, and 11) *Actias selene* (1-5 belong to the family Bombycidae and 6-11 to Saturniidae). The reactivities of their arylphorins to anti-1 (*B. mori*) arylphorin antibody and anti-6 (*S. c. ricini*) were put in order, 1=2>3=4=5=6=7=8=9=10=11, and 6=7>3=8=9=10=11>4=5>1=2 respectively. These results suggest that arylphorins of 3, 4 and 5, which belong to the Bombycidae, are more similar to the arylphorins of Saturniidae rather than those of *Bombyx.*

We also compared the sequences of cDNAs to mRNAs coding for arylphorins of *S. c. ricini* and *B. mori.*

REGIONAL DIFFERENTIATION OF LARVAL FAT BODY IN *Plodia interpunctella* AND RELATED PYRALID MOTHS

P.D. Shirk and C.C. Malone

USDA ARS, Insect Attractants, Behavior and Basic Biology Research Laboratory, Gainesville, FL 32604

Larvae of the Indianmeal moth, *Plodia interpunctella*, and closely related pyralid moths contain two morphologically distinct forms of fat body regionally differentiated along the

anterior-posterior axis: an anterior white fat body morphological similar to previously described fat body and a posterior tan fat body which was previously undescribed. Tan fat body cells have numerous, large, spherical mitochondria that fill most of the cytoplasmic space with only few storage organelles, and this tissue is the predominant fat body during the first three larval instars. In contrast, white fat body cells have organelles primarily involved with storage: large lipid droplets, numerous glycogen granules, and protein granules. White fat body becomes the predominant tissue type during the fourth and fifth (last) larval instars and eventually occupies most of the space of the hemocoel. Tan fat body is apparently a *bone fide* larval fat body as the tissue disintegrates in pharate pupae whereas white fat body undergoes metamorphosis and persists into the adult stage. Both fat bodies produce storage proteins during the late fifth stadium while only the white fat body accumulates storage proteins. The morphology of tan fat body suggests that the larvae have an exceedingly high metabolic capacity and may be able to generate large amounts of metabolic water from seeds. Thus, the presence of tan fat body may provide this moth with an advantage for utilizing grains and dried plant material as food sources. Examination of fat body morphology in other pyralid moths showed that the presence of tan fat body is correlated with the utilization of seeds as a food source.

QUANTITATIVE CHANGES IN STORAGE PROTEIN CONTENT IN TISSUES OF THE WAXMOTH, *Galleria mellonella*, DURING DEVELOPMENT

D. Silhacek, C. Murphy, D. Bean, and C. Malone

USDA ARS, Insect Attractants, Behavior and Basic Biology Research Laboratory, Gainesville, FL 32604

In the waxmoth, storage proteins are largely found in one of two tissues, fat body or hemolymph, depending upon the stage of development and the identity of the storage protein. The arylphorins, 74 kDa and 76 kDa, and the female-predominant, 81 kDa, are synthesized in the larval fat body and are rapidly secreted into the hemolymph where they accumulate, at least during the final four instars. During each larval stadium, titers of these storage proteins in the hemolymph increase during feeding and decline at each molt. The storage proteins appear to be utilized at the larval molts since whole body levels show similar declines. In contrast, the 85 kDa storage protein is first observed to accumulate in the hemolymph during the final larval stadium. At pupation 85 kDa remains in the hemolymph while the other storage proteins are selectively taken up and quantitatively stored as protein granules in the fat body. Two types of protein granules can distinguished by chemical and physical characteristics. All of the storage proteins are utilized during pharate adult development and are largely undetectable in the insect by eclosion.

HORMONAL REGULATION OF OVARIAN DEVELOPMENT IN INDIANMEAL MOTHS

D. Silhacek, G. Zimowska, E. Shaaya, and P.D. Shirk

USDA ARS, Insect Attractants, Behavior and Basic Biology Research Laboratory, Gainesville, FL 32604
ARO, P.O.B. 6, Bet Dagan, Israel

We have correlated changes in ecdysteroid titers with development and function of the ovaries of *Plodia interpunctella* during the pupal stage. Following pupation, the ecdysteroid levels in whole pupae increased to a bimodal peak with maxima of 2000 pg/mg wet weight at 45 h and 60 h. The ecdysteroid titer decreased to ca. 200 pg by 80 h, a level that was maintained until eclosion. During the first day after pupation, the ecdysteroid titer increased to approximately 1000 pg/mg wet weight; concomitantly the larval fat body dissociated.

Ovarian morphology at this time showed the oocyte-nurse cell complexes to be unorganized within the ovarioles. During the second day, the ovarioles began to elongate; the follicles became organized and arranged into a single file array with the most advanced oocytes located closest to the oviduct. The ovarioles continued their rapid growth and elongation through the third day. By the end of the fourth day, the ovarioles contained highly organized follicles, the fat body had reassociated into the adult from and vitellogenin synthesis was initiated by the fat body. Sequestration of YP1/YP3 from the hemolymph and YP2/YP4 from the follicle cells and yolk body formation began on the fourth day and continued through eclosion, with the oocytes rapidly increasing in size. An eclosion, the terminal oocytes still had nurse cells attached and choriogenesis had not been intiated.

WING VIBRATIONS OF WAGGING HONEY BEES DECREASE IN FREQUENCY AS THE DISTANCE TO THE FOOD SOURCE INCREASES

H.G. Spangler

Carl Hayden Bee Research Center, ARS-USDA, 2000 E. Allen Road, Tucson, AZ

Honey bees were trained to a feeding station placed at a series of distances from their hive to study whether the wing oscillation frequency of successful foragers performing the waggle dance changes with increasing distance between the food source and the hive. Higher frequency wing movements were produced by bees foraging near the hive while the frequency dropped at longer distances. For example, the mean frequency was 315 Hz at 50 m while it dropped to 207 Hz at 1600 m. The wingbeat frequency of flying bees also decreased as distance increased. For example, bees flying to a feeding station 50 meters away had a mean wingbeat frequency of 263.4 Hz while, in a separate test, bees flying over 500 m to an unknown destination had a mean wingbeat frequency of 198 Hz. Additional tests revealed that wingbeat frequency of bees flying toward and away from their hive entrance had widely varying wingbeat frequencies which did not correlate consistently with temperature. Thus, the distance a bee flies appears to be an important factor in determining the frequency of wing motion, both while flying and performing the waggle dance. The airborne sound produced by the oscillatory wing movements during the waggle dance may communicate essential information needed by potential recruits before they will leave the hive. That these sounds are correlated with the distance adds another dimension to the type of information available to recruited bees.

CHARACTERIZATION OF THE APOLIPHORIN-III GENE FROM *Manduca sexta*

A.F. Smith, K.D. Cole, and M.A. Wells

Biochemistry Department, Biosciences West, University of Arizona, Tucson, AZ 85721

The sequence of the gene for apolipophorin-III (apoLp-III) from *Manduca sexta*, has been determined. The gene contains four exons: exon 1 contains a relatively short 5′ untranslated region and the first 15 amino acids of the signal sequence; exon 2 contains the remaining 3 amino acids of the signal sequence, the 5 amino acids of the pro sequence and residues of 1-64 of the mature protein; and exon 4 codes for residues 119-165 of the protein

plus the 3′ untranslated sequences. It was shown that the apoLp-III gene is expressed in a cyclic manner during larval development with maximum expression occurring during the feeding period and that adult fat body contains 2-10-fold higher levels of the apoLp-III mRNA. Restriction mapping, cDNA sequencing, and primer extension analysis all indicated that both larval and adult apoLp-III have the same mRNA transcripts and utilize the same coding sequences of a single apoLp-III gene. A putative ecdysone receptor binding site is found approximately 110 nucleotides upstream from the transcription start site. Gel retardation assays show that fat body nuclei from animals which are not expressing the apoLp-III gene contain a protein which binds to the upstream region of the gene. (Supported by NIH grant HL 39116.)

SENSITIVITY OF CORPORA ALLATA TO SYNTHETIC ALLATOSTATIN

B. Stay, A.P. Woodhead, and S. Joshi

Department of Biology, University of Iowa, Iowa City, 52242

The production of juvenile hormone (JH) by corpora allata (CA) is regulated in part by neurosecretory material from the brain. Four allatostatins, peptides that inhibit the synthesis of JH by the CA *in vitro*, have been isolated from the brain of the viviparous cockroach *Diploptera punctata* (Woodhead et al., 1989, Proc. Natl. Acad. Sci. USA 86:5997-6001). Allatostatin 1 is 10x more inhibitory than allatostatin 4 (Woodhead et al., 1989), however, both (tested at 10^{-8} and 10^{-7} M respectively) elicit a similar pattern of inhibition in CA from females at the same stages of the reproductive cycle. CA from first and second cycle females, in which JH synthesis is rapidly increasing (during vitellogenesis), are less sensitive to allatostatins than glands that have lower rates of synthesis (at the completion of vitellogenesis - day 6, virgin females - day 2, and mated females early in pregnancy - day 30). CA from females later in pregnancy (day 50), although low in activity, are not inhibited by 10^{-8} M allatostatin 1 but are strongly inhibited by 10^{-7} M allatostatin 1. The mechanism of differential sensitivity of CA from females of different physiological states remains to be determined.

IDENTIFICATION OF TWO CUTICLE PROTEINS IN AN ESTABLISHED COCKROACH CELL LINE

B. Stiles and R.A. Leopold

USDA, ARS, Bioscience Research Lab, 1605 West College St., Fargo, ND 58105

An embryo-derived cell line from *Blattella germanica*, designated UMBGE-4 (Kurti and Brooks, 1977, In Vitro, 13:11-17), grows in the from of hollow multicellular vesicles which were previously found to synthesize chitin (Marks et al., 1984, Arch. Insect. Biochem. Physiol. 1:225-230). In an attempt to discern whether these cells can synthesize any of the protein components of the cuticle, a polyclonal antibody was prepared using cuticle proteins extracted from *B. germanica* nymphs. When this antibody was used to probe a Western blot of the UMBGE-4 cellular proteins, a protein of approximately 58 kDa molecular weight was observed. If a culture of these vesicle forming cells was first exposed to a regimen of a small followed by a large pulse of 20-hydroxyecdysone, a 34 kDa molecular weight protein was induced which reacted with the anti-cuticle protein antibody. It appears that this cell line is capable of synthesizing at least two nymphal cuticle proteins.

INSECT RESISTANCE TO *Bacillus thruingiensis*

T.B. Stone, S.R. Sims, S.C. MacIntosh, and P.G. Marrone

Monsanto Agricultural Company, 700 Chesterfield Village Parkway, Chesterfield, MO, 63198

Insect resistance to the biological insecticide *Bacillus thruingiensis* (B.t.) has been reported in the laboratory for species of Lepidoptera (McGaughey, 1985, J. Econ. Ent. 78:1089; Stone et al., 1989, J. Invert. Path. 53:228), Diptera, (Golman, et al., 1986, J. Invert. Path. 47:317) and Coleoptera (Whall, personal communication). The development of a strain of *Heliothis virescens* resistant to a genetically engineered *Pseudomonas fluorescens* containing the delta-endotoxin of B.T. subsp. *kurstaki* (HD-1) in our laboratory has led to characterizing the extent of cross resistance among field populations of *H. virescens* and *H. zea* to B.t., and studies to determine the genetic and physiological mechanisms of resistance. Results of these investigations indicate: 1) cross resistance of the *H. virescens* line to more than 25 strains of B.t. representing the subspecies of *aizawai*, *tolworthi*, *kurstaki*, and *darmstadiensis*; 2) variability in response among field populations suggests the potential for resistance development under field conditions; 3) more than one gene is responsible for the development of laboratory resistance; and 4) the physiological mechanism of resistance may result from a decrease in the ability of the endotoxin to bind to the midgut epithelium.

JHIII INFLUENCES THE ACOUSTICAL RESPONSE OF FEMALE CRICKETS, POSSIBLY THROUGH DIRECT EFFECTS ON IDENTIFIED AUDITORY NEURONS

J. Stout, R Walikonis, and G. Atkins

Biology Department, Andrews University, Berrien Springs, MI 49104

The phonotactic response of female *Acheta domestica* to the male's calling song is dependent on adequate levels of juvenile hormone. Removal of the corpora allata eliminated female photaxis, while addition of juvenile hormone III (JHIII) restored the female's phonotactic response within 6 hours. Females became phonotactically responsive to males on the day of or the day following the imaginal molt, 6 to 24 hours following the application of JHIII. Addition of transcription or translation blockers inhibited this JHIII effect. Untreated females typically became phonotactically responsive to males 3 to 5 days following the imaginal molt. More careful evaluation of the onset of phonotaxis by females demonstrated that phonotaxis did not suddenly begin at 4 to 5 days following the imaginal molt. Rather, the behavioral threshold to call song intensity lowered progressively over the first 4 to 7 days following the imaginal molt. During this time JHIII production was increasing in the female. An ascending auditory interneuron (L1) was most sensitive to the carrier frequencies of the calling song, and clearly encoded its temporal structure. The threshold for the response of L1 to the calling song declined progressively over the between days 2 and 7 of adult life in a manner similar to the declining behavioral threshold. During this period, JHIII levels were generally increasing. This suggests a possible regulatory role of JHIII on the threshold for the response of L1 to the calling song which is presently being evaluated. Preliminary evidence also suggested that the main branch of the dendritic branch of L1 increased in size as the animal aged. This structural change may also be influenced by JHIII. Older virgin females (12+days) were less selective for the syllable period of the calling song than were females that were 5 to 7 days old. Application of JHIII to older, unselective females reduced the range of calling song syllable periods that were attractive. Four days following application they were about as selective as young females. By 9 days the females again responded to a broader

range of syllable periods. Another ascending auditory neuron (L3) changed its syllable period processing characteristics in older females (20+days) who, due to their age, would be less behaviorally selective for syllable period. Application of JHIII to these unselective females changed L3's syllable period processing characteristics to the more selective response typical of younger females. These changes in L3 parallel the behavioral changes described above.

MOLECULAR INSECT SCIENCE GOES TO THE FIELD: A MAB-BASED DOT BLOT ASSAY FOR ARTHROPOD PREDATOR STOMACH ANALYSIS

M.K. Stuart and M.H. Greenstone

USDA-Agricultural Research Service, Biological Control of Insects Research Laboratory, P.O. Box 7629, Research Park, Columbia, MO 65205

Ecologists have been using immunological assays for arthropod predator stomach analysis for about four decades. The assay of choice today is ELISA: it is sensitive and, once all of the bugs have been worked out, relatively foolproof. However, ELISAs require expensive spectrophotometric readers to interpret, and a statistical decision to determine negativity or positivity. Our dot blot assay, using a monoclonal antibody to *Heliothis zea* arylphorin, is species- and instar-specific, comparable to ELISA in sensitivity, takes less than three hours to perform, and gives an unequivocally positive or negative result when interpreted by eye. We support these claims with results of assays of homogenates of spiders, wasps and bugs fed the target insect and other noctuids. We hope that rapid, easily interpreted assays such as ours will make immunoassay technology more accessible to arthropod ecologists, thereby increasing our knowledge of the role of arthropod predators in biological control of pests.

THE ATTACINS, THEIR STRUCTURE, FUNCTION AND GENE ORGANIZATION

S.-C. Sun, I. Linström, and I. Faye

Department of Microbiology, Stockholm University, S-106 91, Stockholm, Sweden

Attacins are a group of antibacterial proteins (20 kDa) found in *Hyalophora cecropia*. They are coinduced with a specific set of proteins which are thought to be of absolute importance for the immune response of these insects. These proteins are synthesized and released into the hemolymph after injection of bacteria but can also be induced by injury. Lysozyme and cecropins are other antibacterial proteins induced by the same stimuli in *H. cecropia*. Six forms of atticin have been purified. From amino acid and cDNA sequencing data it was deduced that only two types are gene encoded and these could then be enzymatically altered into new forms. One attacin group is basic and one more acidic. Both forms are active against Gram-negative bacteria. The analysis of several genomic clones from *H. cecropia* reveal a close linkage between one acidic and three basic attacin genes, which are lying in tandem. However, two of the basic attacins genes are pseudo-genes. The basic and the acidic attacin genes have an opposite direction of transcription. Sequence comparisons of the 5´-flanking regions of the attacin genes and other immune genes has shown that they have at least one promoter sequence in common. This sequence could represent a promoter element specific for the immune genes of these organisms. *In vitro* transcription and DNA-protein interaction experiments will give us a better understanding of what factors are important in the control of these genes.

"NON-STICK" MOTH PHEROMONES: SYNTHESIS AND ELECTROPHYSIOLOGICAL ACTIVITY

W.-C. Sun,[1] and G.D. Prestwich,[1] M.S. Mayer,[2] J.C. Dickens[3]

[1] Department of Chemistry, State University of New York, Stony Brook, NY 11794-3400
[2] IABBBRL, USDA/ARS Southern Region, P.O. Box 14565, Gainesville, Fl 32604
[3] USDA Boll Weevil Research Laboratory, USDA/ARS Mid South Region, P.O. Box 5367, Mississippi State, 39762

Perfluoroalkyl analogues of the pheromone constituents of three moths were synthesized and employed for single cell recordings from male antennae. In each analog, the hydrophobic terminus, either a butyl for hexyl sustituent on the (Z)-alkenyl chain, was replaced with a perfluorobutyl (Pfb, C_4F_9) or perfluorohexyl (Pfh, C_6F_{13}) moiety. These perfluoroalkyl analogues showed significantly higher volatility, as demonstrated by a decrease in gas chromatographic retention time by 2 to 4 methylene equivalents in their Kovats retention indices. Sensilla of male *Heliothis zea* responded to 1 μg of Z11-16:A1 and 25 μg of Pfb-Z11-16:A1 with similar firing rates. Negligible post-stimulus firing activity was observed, the perfluoroalkyl groups confer a "slipperiness" to the pheromone analogues. That is, these fluorinated analogues show a weaker interaction between the hydrophobic protein binding site and the more rigid and polar perfluoroalkyl moiety.

CONSTRUCTION OF A cDNA LIBRARY FROM CABBAGE LOOPER MOTH SEX PHEROMONE GLANDS

J.D. Tang, W.L. Roelofs, and D.C. Knipple

Department of Entomology, New York Experiment Station, Cornell University, Geneva, NY 14456

A directional cloning procedure was adapted to construct a cDNA library from cabbage looper moth sex pheromone glands. Pheromone gland poly(A)$^+$RNA was isolated by guanidinium/phenol extraction followed by oligo-dT cellulose chromatography. Reverse transcriptase-mediated first strand cDNA synthesis of the mRNA template was primed with an oligonucleotide adaptor which had a 3´ oligo-dT tail and a 5´ *Eco*RI recognition sequence. The first strand cDNA was then G-tailed at the 3´ end with terminal transferase prior to vector insertion. The vector, lambda Zap, was cut in the polylinker region with SpeI and ligated to a double stranded oligonucleotide adaptor containing a protruding 5´ oligo-dC tail. A unique *Eco*RI site, also in the polylinker region, was then used to remove the ligated adapters from one arm of the vector. After ligation of the first strand cDNA to the vector, *E. coli* DNA polymerase I and *E. coli* DNA ligase were used to fill in the gap. The DNA was then packaged and plated.

CHEMICAL AND BIOCHEMICAL STUDIES OF ALDEHYDE OXIDASES AND DEHYDROGENASES IN *Heliothis* MOTH ANTENNAE

M.L. Tasayco and G.D. Prestwich

Department of Chemistry, State University of New York, Stony Brook, New York, 11794-3400

Aldehyde oxidase (AO) and aldehyde dehydrogenase (ALDH) enzymes have bee identified in polyacrylamide gel electrophoresis (PAGE)-separated soluble proteins from tissues of both male and female moths in genus *Heliothis* (Lepidoptera: Nocuidae). AOs are visualized using horseradish peroxidase to detect the hydrogen peroxide produced during oxygen-requiring aldehyde oxidation. ALDHs can be visualized using [^{3}H]-vinyl ketone, i.e., chemically reactive analogues on Z11-16:A1 which specifically modify active site sulfhydryl groups. In solution, AO and ALDH activities can be measured using radio-TLC and spectroscopic assays with [^{3}H]-labeled and unlabeled Z11-16:A1, respectively. Six AO antenna-specific isozymes have been identified by IEF with pI values between 4.6 and 5.3. The most abundant ALDH isozyme in both heads and antennae shows a size of 50 kDa and characterization of *H. virscens* head ALDH, labeled covalently with [^{3}H]-Z1,11-16:3-oxo, is described.

IDENTIFICATION OF HEMOLYMPH VITELLOGENIN IN THE COTTON-BOLL WEEVIL

T.E. Taub and M.A. Rankin

Department of Zoology, University of Texas at Austin, Austin, TX 78712

The cotton-boll weevil is a serious agricultural pest which causes millions of dollars of crop loss each year, yet little is known of its reproductive physiology. Indeed, with the exception of *Leptinotarsa decemlineata*, knowledge of the endocrine control of reproduction in Coleoptera in general is sadly lacking. We have begun an analysis of differences in hemolymph proteins of reproducing and diapausing boll weevils using SDS-PAGE. Results from SDS-PAGE and Western blot transfer demonstrate that vitellogenin is not present in the hemolymph of female weevils during diapause. Vitellogenin production can be restored by placing diapausing female weevils into an environment which induces reproduction. Preliminary evidence suggests that although JH-esterase levels are elevated during diapause topical application of JH to diapausing females does not induce vitellogenin synthesis. Work is in progress to determine the endocrine control of vitellogenin synthesis.

SINGLE CHANNEL STUDIES OF GABA RECEPTORS ON INSECT NEURONES

A. Taylor and D. Beadle

School of Biological and Molecular Sciences, Oxford Polytechnic, Gipsy Lane, Headington, Oxford OX3 OBP, U.K.

GABA receptors on freshly disassociated locust neurones and cultured cockroach neurones have been studied using the patch clamp technique in both the cell-attached and whole-cell voltage-clamp models. Application of GABA and other agonists to these neurones held under whole-cell voltage-clamp conditions evoked an inward current that was accompanied by an increase in current noise and this noise has been analyzed by fluctuation analysis to obtain data on mean channel open time and conductance. Fluctuation analysis has been used to investigate the action of antagonists and modulators. Preliminary single channel data has

been obtained for GABA, muscimol and ZAPA by either inclusion of these agonists in the patch electrode of by application ot outside out patches. A combination of the noise analysis and single channel data provides detailed information on the properties of these receptors and their associated ion channels.

EFFECT OF HYBRIDIZATION ON SEX PHEROMONE BIOSYNTHESIS IN *Heliothis* MOTHS

P.E.A. Teal and J. Tumlinson

USDA/ARS/Insect Attractants Behavior and Basic Biology Research Laboratory, 1700 S.W. 23rd Drive, Gainesville, FL 32604

Enzymatic steps which regulate production of oxygenated functional moieties in the pheromones of *Heliothis* species include cellular acetate esterases and cuticular alcohol oxidases. The species specific activities of these enzymes establish both the types of compounds released as pheromones and their ratios. Analysis of pheromone gland extracts obtained from females of reciprocal F_1 hybrids between *H. virscens* and *H. subflexa* indicated that 25-30% of the females has acetate in the gland but the amount was less than 1% of the major pheromone component. This ratio was different from both parent species. The ratio of alcohol to aldehyde in both F_1 hybrid groups was the same as that of *H. virescens* females. Backcrossing females of either F_1 group to *H. subflexa* males yielded females which always produced acetate but the ratio of acetate to aldehyde was much lower than that of the paternal species. Backcrossing to *H. virescens* males resulted in females having the same acetate:alcohol phenotype as the paternal species. Alcohol:aldehyde rations for females obtained from backcrossing the *H. virescens* males were the same as that of *H. virescens.* Twenty five percent of females obtained from backcrossing either F_1 hybrid to *H. subflexa* males had rations of alcohol to aldehyde which are like *H. subflexa*. The remainder had the same ratio that *H. virescens* females maintain. These data indicate that esterase activity in the hybrid and backcrossed insects is not regulated by simple autosomal dominance of the *H. virescens* genome. However, the data suggest that oxidase activity is regulated by two autosomal loci with the *H. virescens* phenotype being epistatic to that of *H. subflexa*.

MOLECULAR ANALYSIS OF EMBRYONIC DEVELOPMENT IN A SHORT GERM INSECT, *Schistocerca gregaria*

G. Tear, M. Akam, and A. Martinez-Arias

Departments of Genetics and Zoology, Cambridge University, Cambridge, U.K.

During embryonic development all insects develop a basic metameric body plan consisting of a protocephalon with 3 gnathal, 3 thoracic, and 8-11 abdominal segments and a telson. There are two distinct developmental mechanisms used to generate this "ground-state". Long germ insects determine all their segments simultaneously by dividing the blastoderm into a series of metameric units through the action of the segmentation genes (Akam, M., 1987, Development 101:1-22; Ingham, P.W., 1988, Nature 335:25-34). Short germ insects form a blastoderm which consist only of protocephalon and telson plus a region that will grow to give rise to the segmented portion of the animal. As the sub-terminal region grows through cell division the segments are formed in an anterior to posterior progression. We would like to know whether segmentation genes similar to those used by *Drosophila melanogaster* are utilized by the short germ insect and if so how they interact with the process of growth in the production of the body plan. Many of the *D. melanogaster* genes involved in embryonic development contain a conserved sequence of 180 base pairs termed the homeo-box. Using

homeo-box probes from *D. melanogaster* I have isolated and characterized a *Schistocerca genaria* genomic clone which is a homologue of the *D. melanogaster abdominal-a* gene. Using tissue *in situ* hybridization we have demonstrated the pattern of transcription of this gene and of a homologue of *Sex* combs reduced. We have also produced a polyclonal antibody to the *abdominal-a* homologue. Using this antibody we have shown the spatial and temporal expression pattern of the protein product during the embryonic development of *S. genaria*. The genes are expressed in regions corresponding to their homologues in *D. melanogaster*. These results show that homologues of *D. melanogaster* homeotic genes are present in other insects and provide molecular evidence that parasegments are important in the development of insects other than *Drosophila*.

DIETARY GLUCOSE REPRESSES RNA LEVELS FOR ALPHA-AMYLASE AND A NON-HOMOLOGOUS GENE LINKED TO THE AMY LOCUS IN *Drosophila melanogaster*

D.B. Thompson and W.W. Doane

Arizona State University, Tempe, AZ

In *Drosophila melanogaster*, the duplicated, divergently transcribed structural genes for alpha-amylase, *Amy-p* (proximal) and *Amy-d* (distal) are located about 4 kb apart in polytene map region 54A of chromosome *2R*. The enzyme is produced primarily in the midgut. Dietary glucose is known to reduce the levels of amylase activity, amylase protein and translatable amylase message in larval or adults or both. In addition, the repressive action of glucose on amylase mRNA abundance has been shown for whole larval extracts (Benkel and Hickey, 1987, Proc Nat Acad Sci USA, 84:1337). While using Northern analysis to study dietary glucose repression of amylase RNA levels, a previously unknown transcription unit (TU) was discovered that is not homologous to *Amy* but whose RNA level is also repressed by glucose in the food medium. The new TU is located centromere-proximal to *Amy-p* at an estimated distance of 2.0 kb. It is transcribed in both strains of *D. melanogaster* examined and its RNA appears in larval and adult stages. Its RNA is shorter than that for amylase and measures about 1500 bases long. Of the *Eco*Rl fragments subcloned into pBT vectors from the *Amy* locus clone Dm65, only the 2.8 kb fragment hybridized with the RNA of this new TU. This 2.8 kb fragment was sequenced and from a computer search of the data, its only open reading with *D. melanogaster* condon usage bias is a 150 bp sequence at its proximal end. The latter appears to represent the 3′ end of the newly identified TU, most of the gene extending beyond the insert in Dm65. Overlapping clones containing the remainder of the putative TU are under analysis, as is a cDNA which appears to be homologous to it. Although no genes with high sequence similarity to the 150 bp sequence of this TU were revealed by computer searches, a human glucose transport protein with moderate sequence similarity has attracted our attention.

EFFECT OF ECDYSONE ON *In vivo* PROTEIN METABOLISM OF DEVELOPING FLIGHT MUSCLE OF THE TOBACCO HORNWORM, *Manduca sexta*

M.E. Tischler, P. Cook, S. Hodsden, M. Wu, and S. McCready

Department of Biochemistry, University of Arizona, Tucson, AZ

Methodology had been developed to study *in vivo* protein metabolism in the dorsolongitudinal flight muscle during the final 4 days of adult development. These final 4 days have been subdivided into stages characterized morphologically by Schwartz and Truman. We have timed the duration of stages 6-12 to obtain growth rates of 150, 130, 60, 80, 50, 50, and 5 μg protein/h in the successive stages. The negligible rate in the final stage showed that

growth of the muscle ceased about 24 h before eclosion. 20-Hydroxyecdysone (20-HE) increased growth markedly during Stages 10 and 11, somewhat in Stages 8 and 9, and not in Stage 12. The maximal dose was about 5 μg/g insect. *In vivo* protein synthesis was measured in two ways using either a "flooding technique" where 5 μCi and 30 μmol of [^{3}H]phe was injected, or injection of tracer amount of [^{3}H]phe only. In the former method it is possible to estimate protein synthesis from a single time point at 1.5 to 2 h after injection since equilibration of radioactivity in the hemolymph occurs within 7 min and the muscle specific activity remains constant for at least 2 h. The latter method relies on measuring linear incorporation of radioactivity and estimating turnover of the radiolabeled phe pool. Since the two methods give similar results, the former method is preferred because of its simplicity. Using measured *in vivo* rates of protein synthesis and growth it is possible to calculate *in vivo* protein degradation as synthesis - growth. These calculations showed a high rate of both protein synthesis and degradation, and that the decline in muscle growth was due to diminished protein synthesis. 20-HE decrease protein synthesis in some stage and produced this effect within 4 h. Thus 20-HE must promote muscle growth restricting the rate of protein degradation. (Supported by NSF grant 87-12458 and a fellowship to S.H. from and NSF center grant to the Center for Insect Science, Univ. of Arizona.)

CHARACTERIZATION OF IMMUNE COMPETENT HEMOCYTES BY MONOCLONAL ANTIBODIES

T. Trenczek,[1] H. Bennich[2]

[1] Institute of General Zoology, Königin-Luise-Str. 1-3, D-100o Berlin
[2] Institute of Immunology, Box 582. S-751 23 Uppsala, Sweden

Bacterial infections as well as injury induce cellular and humoral defense mechanisms in *Hyalophora cecropia*. Humoral factors, the so called "immune proteins" P4, attacins, lysozyme and cecropins (Boman & Hultmark, 1987, Ann. Rev. Microbiol. 41:103-106) are shown to be synthesized by the fat body. During studies with fat body *in vitro* cultures it became obvious that hemocytes also are able to synthesize the "immune proteins". As shown by immunohistochemistry the number of immune-positive cells after induction varies between 90-100% (in case of infection) and 30-90% (in case of injury) depending on the developmental stage and the protein investigated. However, already a small number of cell from naive pupae or larvae show a positive reaction with the appropriate antibody. Since it was not possible to correlate definitely the ability to synthesize the "immune proteins" to certain cells of the granulocyte-plasmatocyte population, monoclonal antibodies (mabs) were prepared against hemocytes of *Hylophora cecropia*. All mabs finally selected show distinct binding activity only to certain percentages of hemocytes indicating different subpopulations of cells. Further investigations have to clarify if the characterization of hemocyte subpopulations by mabs follows the characterization based on morphological criteria and if the ability to synthesize "immune protein" is linked to certain subsets of hemocytes.

Financial support by the Swedish Medical Research Council, the Gunnar Hannson Forskingsstiftelse and the DFG is gratefully acknowledged.

cDNA AND GENOMIC CLONES FOR A TESTIS-SPECIFIC PROTAMINE-LIKE PROTEIN FROM THE BOLL WEEVIL, *Anthonomus grandis*

P.M. Trewitt,[1] L.J. Heilmann,[2] S.S. Degrugillier,[2] and A.K. Kumaran[1]

[1] Department of Biology, Marquette University, Milwaukee, WI
[2] USDA-ARS Biosciences Research Laboratory, Fargo, ND

As a first step to developing safer methods of insect control, we have begun a study of sex-specific gene expression in the boll weevil, *Anthonomus grandis*. From an adult male library, we isolated a cloned cDNA expressed only in the testes. The nucleotide sequence of the 745 bp cDNA contains a 396 base open reading frame coding for an arginine-rich protein with similarity to vertebrate protamines. Initiation of translation is thought to occur at the fourth ATG from the 5′ end of the cDNA. The primary translation product is much larger than the vertebrate protamines and has two arginine-rich regions separated by a long central spacer region. Genomic Southern blots show a complex pattern of hybridization with the cDNA, indicating that the sequence is encoded by a small gene family. Two distinct genomic clones have been isolated from an EMBL3 library and portions hybridizing with the protamine cDNA mapped and subcloned. Sequencing revealed that these genomic fragments differ at less than 1% of their bases and code for identical proteins. Each gene contains two small introns. Work is in progress to isolate and characterize the sex-specific and tissue-specific promoter regions of these genes.

PURIFICATION AND PROPERTIES OF EGG SPECIFIC PROTEIN FROM *Manduca sexta*

K. Tsuchida, J. Kawooya, J.H. Law, and M.A. Wells

Department of Biochemistry, BioSciences West, University of Arizona, Tucson, Az 85721

Egg specific protein (ESP) has been isolated from the eggs of *Manduca sexta*. ESP was isolated by a combination of density gradient ultracentrifigation , DEAE ion-exchange chromatography, gel filtration and hydroxyapatite chromatography. The purified protein is a dimer of M_r 65,000 apoprotein, a native M_r 140,000 species was obtained by gel permeation chromatography and native acrylamide gel electrophoresis. The holoprotein contains lipid and carbohydrate. Western blotting and immunodiffusion showed ESP was present only in follicle or ovary, not in hemolymph, fat body or testes. (Supported by NIH grant HL-39116)

CHARACTERIZATION OF LIPOPHORIN RECEPTORS FROM THE FAT BODY OF *Manduca sexta*

K. Tsuchida, and M.A. Wells

Department of Biochemistry, BioSciences West, University of Arizona, Tucson, Az 85721

A lipophorin receptor has been purified from the fat body of *Manduca sexta* larvae. The purification involves solubilization of membrane proteins in detergent, DEAE and hydroxyapatite chromatography, affinity chromatography on a concanavalin A column, and affinity chromatography on a lipophorin-sepharose column. An overall purification of 220-fold from the solubilized membranes was achieved. The receptor has a molecular mass of 120 kDa. The receptor has an absolute requirement for Ca^{2+} and is inhibited by suramin. The pH optimum of the receptor is 6.5, which is near the pH of the hemolymph. Binding

indicate a single high affinity binding site with a Kd = 4.1 ± 0.19 x 10^{-8} M as measured with the lipophorin isolated from larval hemolymph. It was shown that the affinity of the receptor for lipophorin varies directly with the diacylglycerol content. It is proposed that the decrease in affinity of the receptor for lipophorins depleted of diacylglycerol plays a key role in facilitating the transport of diacylglycerol from the midgut to the fat body during the larval feeding period. The insect receptor has some properties which are similar to those of vertebrate lipoprotein receptors, *viz*, molecular weight, requirement for Ca^{2+}, and inhibition by suramin. However, the insect receptor does not bind vertebrate lipoproteins. (Supported by NIH grant HL-39116.)

IDENTIFICATION OF A LIPOPHORIN BINDING SITE ON THE FAT BODY OF THE MIGRATORY LOCUST, *Locusta migratoria*

R. Van Antwerpen,[1] H.J. Wynne,[2] D.J. Van der Horst,[1] and A.M.T. Beenakkers[1]

[1] Department of Experimental Zoology
[2] Center for Biostatistics, University of Utrecht, 8 Padualaan, 3584CH Utrecht, The Netherlands.

We studied the interaction of locust lipophorins with intact pieces of fat body tissue, using a radiolabelled ligand binding assay. At 33°C, binding of [^{3}H]-HDLp was shown to correlate linearly with tissue concentration up to ±7 mg of fat body protein per ml of incubation medium. The [^{3}H]-HDLp binding that was displaceable by a 20-fold excess of unlabelled HDLp (which is an approximation of the specific binding) reached equilibrium after ±2 h; low levels of non-displaceable binding increased linearly during this time interval. Specific binding was determined by fitting the total binding data (specific plus non-specific binding) to a mathematical model of a single high affinity binding site plus non-specific low affinity binding. Thus the presence of a single [^{3}H]-HDLp binding site with an equilibrium dissociation constant of Kd = 3.1 (±0.5) ng.ug-1 tissue protein was detected on the fat body. Competition experiments demonstrated that the affinity of unlabelled HDLp for the binding site is similar to the affinity of [^{3}H]-HDlp. Unlabelled low density lipophorin (LDLp), however, appeared to have a ±20-fold lower affinity for the binding site. It is suggested that the identified binding site represents a lipophorin receptor which may be involved in the formation of LDLp during sustained locust flight.

(RVA, present address: Department of Biochemistry, Biological Sciences West, University of Arizona, Tucson, Az 85621

BIOCHEMICAL AND GENETIC CHARACTERIZATION OF FIRE ANT POPULATIONS IN THE UNITED STATES AND SOUTH AMERICA

R.K. Vander Meer,[1] S. Narang,[1] S. Toniolo,[2] and R.S. Patterson[1]

[1] USDA/ARS, P.O. Box 14565, Gainesville, Fl 32604
[2] Department of Entomology and Nematology, University of Florida, Gainesville, Fl 32611.

The imported fire ants, *Solenopsis invicta* and *Solenopsis richteri*, create medical and agricultural problems in the 130 million hectares they infest in the united states. Classical taxonomy of these two species was complicated by the formation of reproductively viable hybrids. The hybrid was discovered using species-specific venom alkaloid and cuticular hydrocarbon biochemical characters, later hybridization was verified through isozyme studies on female sexuals. We report alkaloid, hydrocarbon and genetic evidence (using readily

accessible worker fire ants) that demonstrates the presence of two cryptic *S. richteri* species in Argentina and readily distinguishes *S. invicta, S. richteri* and their hybrid in the United States. The data suggest a simple explanation for the lack of hybridization in Argentina and hybridization in the United States.

LIPID EXCHANGE BETWEEN FAT BODY AND LIPOPHORIN: THE ROLE OF A LIPID TRANSFER PARTICLE

M.C. Van Heusden and J.H. Law

Department of Biochemistry, University of Arizona, Tucson, Az 85721

The main lipoprotein in insects is the high density lipophorin or HDLp. This lipophorin functions as a reusable 2shuttle in the transport of a wide range of lipids between gut, fat body and peripheral tissues. Thus lipophorin is a very dynamic particle, continuously loading and unloading lipid. By radiolabeling lipid (mainly diacylglycerol, DG) in either fat body or HDLp, we were able to show *in vitro* a continuous bidirectional exchange of DG between fat body and HDLp of *Manduca sexta*. A lipid transfer particle (LTP), a macromolecular very high density lipoprotein itself, has been isolated from *M. sexta* hemolymph and has been shown to facilitate transfer of lipids between lipoproteins *in vitro*. Using polyclonal antibody against LTP, we showed that some LTP, present at the fat body, catalyzes DG transfer from fat body to HDLp (however not in the opposite direction). In some insects, flight activity induces the conversion of HDLp into a low density form, LDLp, by lipid loading at the fat body. The increased lipid load is stabilized by the binding of several molecules of a small apoprotein, apoLp-III. the newly formed LDLp enables an increased supply of DG to the active flight muscle. Although the mechanism of LDLp formation is largely unknown, we showed the involvement of LTP in this process: during *in vitro* incubation of fat body with HDLp, apoLp-III and adipokinetic hormone, LDLp was formed only after the addition of a catalytic amount of LTP. This result indicates a crucial role of LTP in lipid mobilization during flight and is the first report of lipid transfer from cell to lipoprotein mediated by a lipid transfer particle. (Supported by NIH GM 29238.)

TEMPORAL CONTROL OF ORNITHINE DECARBOXYLASE GENE EXPRESSION DURING EMBRYONIC DEVELOPMENT OF *Musca domestica*

D. Vaske, S.L. Lilleberg, S.K. Killilea, R. Leopold, and R.B. Sparks

Biochemistry Department, NDSU and Insect Genetics, USDA Biosciences Lab, Fargo, ND 58105

Ornithine decarboxylase (ODC) is the first enzyme in the biosynthetic pathway of polyamines and is rate limiting. ODC activity is strictly regulated at several levels: synthesis, degradation, and post-translational activation/deactivation. Cell growth is usually accompanied by high ODC activity, and sufficient levels of polyamines are required for growth-related functions and for chromatin stabilization. We have investigated the temporal order of regulation of ODC gene expression during embryonic development of *Musca domestica*. We have found by Northern blot analysis that ODC specific mRNA first appears mid-way through development and is synthesized continuously at high levels until the eggs hatched. Enzymatic assay of ODC throughout embryogenesis showed that active ODC is present only at the end of embryogenesis just prior to hatching which is about six to seven hours after the first ODC mRNA is synthesized. We found that the inactive embryonic ODC is associated with the plasma membrane and can be released as an active enzyme by activators of G-proteins and phosphatidylinositol-specific phospholipase C. These result suggest that ODC activation may be regulated through a G-protein like signal transduction mechanism.

MATHEMATICAL SIMULATION OF THE ACTIVATION OF FAT BODY GLYCOGEN PHOSPHORYLASE AND TREHALOSE SYNTHESIS BY PEPTIDE HORMONES IN THE AMERICAN COCKROACH

J.A. Veenstra

Department of Entomology, University of Arizona, 434 Forbes, Building #36, Tucson, Az 85721

A model is described which simulates activation of glycogen phosphorylase and induction of trehalose synthesis in the fat body of the American cockroach, *Periplaneta americana*, by two hypertrehalosaemic peptides. Parameters for the model were estimated from literature data (Siegert et al., Insect Biochem. 16:365), with the exception of the half life of the physiologically active peptides, which was estimated from the model. The model describes satisfactorily the activation of glycogen phosphorylase and the increase of haemolymph carbohydrates, which is dependent on the activation of glycogen phosphorylase in the model. It further explains the observed differences in sensitivity for glycogen phophorylase activation and increase in haemolymph carbohydrates by these peptides. Best fits were obtained with a physiological half life of about 12 to 15 minutes for the peptides. This value is similar to what can be calculated from the in vivo effects of these peptides on heart beat (Gersch et al., Zool. Jb. Physiol. 86:17), but it is considerably shorter than the published half life of one hour for radioactive peptide (Skinner et al., Insect Biochem. 17:433). However, both values are compatible, if part of the peptides in the haemolymph are not present in freely dissolved form, but bound to a haemolymph component. The model half life would then represent the half life of the free, physiologically active peptide, which was estimated from the disappearance of radioactive peptide to be about 12 to 15 minutes. This suggests, that the model half life is realistic and physiologically more important than the half life of radioactive peptide of one hour.

PROTEIN PROFILE IN THE FAT BODY, HAEMOLYMPH AND OVARY OF *Laccotrephes griseus*: INFLUENCE OF BIOCIDE *Bacillus sphaericus* (STRAIN 1593)

A. Velpandi [1] and S. Mathavan[2]

[1] School of Biological Sciences, Madurai Kakaraj University, Madurai 625 021, India
[2] Present address: Department of Entomology, University of California, Davis, CA 95616

Protein in the fat body, haemolymph and ovary of 10, 20 and 30 days old adult female *Laccotrephes griseus* was profiled by SDS-PAGE. Similar observations were made in *L. griseus* exposed to the biocide concentrations of 10, 50 and 100 μg/ml for the period of 10, 20 and 30 days. At the lowest concentration of biocide, irrespective of the exposure duration, there were no significant changes in the protein profiles in the haemolymph or fat body. However, marked reductions were observed in the higher concentration of biocide. Combination of increased concentration of biocide in the medium and longer duration of exposure resulted in the reduced synthesis of proteins in the fat body and ovary. The mechanism of influence of biocide in the protein synthesis of *L. griseus* is discussed.

ENGINEERING OF NOVEL BACULOVIRUS VECTORS FOR THE EXPRESSION OF FOREIGN GENES IN INSECTS

J.M. Vlak, A. Schouten, P.W. Roelvink, J.W.M. Martens, and D. Zuidema

Department of Virology, Agricultural University, Binnenhaven 11, 6709 PD Wageningen, the Netherlands

Baculovirus are attractive biological control agents of insect pests in agriculture and forestry. They are also exploited as biotechnological tools for high-level expression of foreign genes in a eukaryotic environment. Baculoviruses contain two genes, that are expressed abundantly late after infection: polyhedrin, coding for the matrix protein of polyhedra, and p10, the function of which is unknown. Polyhedra protect the virus from decay in the abiotic environment and are essential for the initiation of infection in insects. The p10 protein is not required for virus replication, polyhedron formation or insecticidal activity. Introduction of foreign genes into baculoviruses under the control of the p10 promoter is therefore attractive for production of recombinant proteins in insects. However, the isolation of p10 recombinants is difficult, since there is no easy screening or selection system, in contrast to polyhedrin recombinants which can be detected visually by their polyhedrin-negative appearance. We have now developed a novel and convenient vector system for the selection of p10 recombinants for the expression of foreign genes. A special transfer vector was designed containing the flanking regions of the p10 gene to enhance recombination, a unique cloning site for the insertion of the gene of choice, and a special reporter gene cassette. The cassette contains a LacZ gene under the control of a heat-shock promoter. Recombinants containing a foreign gene can now be conveniently selected by the expression of the reporter gene (beta-galactosidase). This screening system is also less dependent of a high frequency of recombination, due to the sensitive detection system. A major drawback to a wide-spread use of baculoviruses as control agents is their slow speed-of-action. Upon infection insects stop feeding only after a few days, where immediate action is required. Genetic manipulation of these viruses by introduction of genes for insect toxins, hormones or metabolic enzymes may increase the speed of action. This novel screening system has been used to select baculovirus recombinants expressing *Bacillus thuringiensis* delta-endotoxin and juvenile hormone esterase.

STUDIES ON DNA POLYMERASE OF SILKWORM NUCLEAR POLYHEDROSIS VIRUS

S.N. Voronova, K.A. Marlyev, P.K. Kullyev, and V.S Mikhailov

N.K. Koltzov Institute of Developmental Biology USSR Academy of Sciences, Vavilov str. 26, Moscow 117808, USSR

Nuclear polyhedrosis virus (NPV) of the silkworm *Bombyx mori* contains a circular double-stranded DNA genome of 125 kbp. After injection into the silkworm pupae BmNPV induces the synthesis of early viral proteins, including a virus-specific DNA polymerase, and late viral products, including polyhedrin. The viral DNA polymerase (BmNPV Pol) was purified from the infected pupae to near homogeneity. The enzyme consists of a single, catalytically active 105 kDa polypeptide. BmNPV Pol shows the properties of a DNA-dependent DNA polymerase and does not use polyribonucleotides as templates. The enzyme possesses the intrinsic 3´ to 5´ exonuclease activity which presumably performs proofreading

in the course of the virus replication. The mode of hydrolysis of single-stranded DNA by BmNPV Pol was distributive: the enzyme dissociated from ssDNA after the release of a single dNMP and then reassociated with the polynucleotide being degraded. The mode of hydrolysis of double-stranded DNA was processive or distributive depending on the primary and the secondary structure of the duplexes. During elongation on single-stranded DNAs the enzyme was highly processive. The secondary structures in DNA templates arrested the driving of BmNPV Pol along the templates and caused pauses in polymerization, indicating the enzyme has a low ability for synthesis with strand displacement. Several nucleotide analogues, including 3´-modified nucleotides, appeared to be potent and specific inhibitors of the viral DNA polymerase.

ARACHICONIC ACID METABOLISM IN THE MOSQUITO, *Aedes aegypti*

M. Vundla and G.J. Blomquist

Department of Biochemistry, University of Nevada, Reno, Nv 89557-0014

The mosquito, *Aedes aegypti*, requires a dietary source of arachidonic acid or related polyunsaturated fatty acid. Consistent with this requirement, labeled acetate injected into the mosquito was incorporated only into saturated and monounsaturated fatty acids. Eighteen carbon mono-, di- and tri-unsaturated fatty acids were not further desaturated nor elongated. Radioactivity from arachidonic acid injected into the hemocoele was recovered in the thorax (50%), head (21%), cuticle related tissue (19%), ovary (6%) and fat body (4%). Most of the radioactivity was in the phospholipid fraction and all of the radioactivity recovered in the phospholipids and acylglycerols stayed as arachidonic acid. In addition to being recovered in esterified lipid, a significant portion of the injected arachidonic acid was converted to products that co-eluted with 15-hydroxyeicosatrienoic acid (HETE) and material intermediate in polarity between HETEs and arachidonic acid. Data on the structures of these eicosanoids and the conversion of labeled arachidonic acid to more polar metabolites will be presented.

GENOME ORGANIZATION AND COMPLEXITY OF THE MOSQUITO, Aedes aegypti

A. Warren, K. Gale, and J. Crampton

Wolfson Unit of Molecular Genetics, Liverpool School of Tropical Medicine, Liverpool L3 5QA, U.K.

The genome of *Aedes aegypti* is both large and complex, being one third the size of the human genome, and exhibits a short period interspersed repeat pattern. We describe the use of DNA reassociation kinetics to determine the total genome size and complexity together with the individual complexity and copy number of the unique, middle and highly repetitive repeated DNA fractions of *A. aegypti* cell line and larval DNA. The implications of patterns of sequence arrangement and genome complexities for experiments aimed at isolating specific classes of DNA sequences, such as mobile genetic elements, are discussed.

TRANSPOSABLE GENETIC ELEMENTS IN THE GENOME OF THE MOSQUITO, *Aedes aegypti*

A. Warren and J. Crampton

Wolfson Unit of Molecular Genetics, Liverpool School of Tropical Medicine, Liverpool L3 5QA, U.K.

Aedes aegypti the major urban vector of yellow fever, dengue and dengue haemorrhagic fever, is the object of control measures on an immense scale. Many of the existing control methodologies are thwart with problems and it is envisaged that manipulation of the *A. aegypti* genome may eventually provide a novel means for controlling this vector population or its ability to transmit pathogens. Transposable genetic elements (TGEs) have been successful in genetic manipulation of a number of organisms. In the same way, indigenous TGEs from *A. aegypti* may prove to be useful tools for the manipulation and characterization of this mosquito genome. Using a number of parallel approaches, we have isolated several *A. aegypti* sequences sharing biochemical and structural properties with previously characterized eukaryotic retrotransposons. Evidence will be presented for the presence of retroposon-like elements within the *A. aegypti* genome. Their potential as tools for characterization and manipulation of the *A. aegypti* genome, with a view to enhancing existing control methods, will further be discussed.

ANTI-VENOM GLAND ANTIBODIES NEUTRALIZE *Campoletis sonorensis* VIRUS

B.A. Webb and M.D. Summers

Department of Entomology, Texas A&M University, College Station, Tx 77840

Venom gland proteins and *Campoletis sonorensis* virus (CsV) structural proteins are immunologically related. The relationship between the structure and function of the conserved epitopes in venom and viral proteins is unclear. To study the role of these conserved epitopes in infection and expression of CsV in parasitized *Heliothis virescens* larvae a neutralization assay has been developed. Following natural parasitization or injection of purified CsV, *H. virescens* larval growth and development is inhibited. Viral neutralizing activity was indicated when viral effects on growth and development were blocked by the test antibody. Monoclonal antibodies which recognized conserved viral and venom epitopes neutralized CsV activity while antibodies which recognized other epitopes did not have neutralizing activity. Temporary immunity to natural parasitization could be induced by injection of anti-venom gland monoclonal antibodies before parasitization. Viral proteins which contain the conserved epitopes have been localized to the membrane fraction of CsV. The presence of immunologically related gene products in CsV and the venom gland may be indicative of a conserved function for these proteins and it may also reflect the structural organization of CsV in which viral gene families that are expressed in parasitized *H. virescens* larvae have been discovered.

NEUROHEMAL ORGAN FORMATION IN THE TOBACCO HORNWORM, *Manduca sexta*

A.L. Westbrook, N. Agui, and W.E. Bollenbacher

Department of Biology, CB #3280, Coker Hall, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-3280

The prothoracicotropic hormone (PTTH) in the tobacco hornworm, *Manduca sexta,* is produced by neurosecretory cells in the procerebrum and is released from the neurohemal terminals in the corpora allata. These neurosecretory cells, the L-NSC III, differentiate early during the embryonic development of *M. sexta*. Their axons grow along specific pathways in the brain before exiting via the nervi corporis cardiaci I+II and growing to the corpora allata. Only when the axons reach the corpora allata do they begin to arborize, suggesting that the corpora allata may provide the stimulus for axonal branching. However, if fifth instar larvae are allatectomized by severing the retrocerebral nerve, the axotomized L-NSC III endings will regenerate and arborize to form a neurohemal-like structure. Furthermore, these regenerated structures appear to be functional for PTTH release since larval-pupal development continues. In this instance, the stimulus for axonal branching and neurohemal organ formation appears to be something other than the corpora allata. Thus, the formation of the L-NSC III neurohemal organ during development may not depend upon the corpora allata, but rather some other feature of the axonal microenvironment that is retained during postembryonic development.

ULTRASTRUCTURAL AND IMMUNOCYTOCHEMICAL OBSERVATIONS ON THE EJACULATORY DUCT AND THE FORMATION OF SPERMATOPHORE IN THE MALE *Tenebrio molitor*

F. Weyda, J.L. Schuette, and G.M. Happ

Zoology Department, Marsh Life Science Bldg., University of Vermont, Burlington, VT 05405

The complex spermatophore of the mealworm beetle is derived from may components. The anterior part of the spermatophore containing sperm cells is composed of two main structures, the outer wall and the core, each of which is multilayered. Sperm cells are located in the lumen between these structures. The central zone of the core is filled with vacuolar-like secretion. The same secretion covers the whole anterior part of spermatophore. Several monoclonal antibodies developed in our laboratory recognize epitopes in secretions of the accessory glands. We have used immunochemical techniques (peroxidase and colloidal gold) to localize these antigens. Monoclonal PL 3.4 identifies antigens in the vacuolar secretion on the surface and in central core of spermatophore. Monoclonals PL 6.3 an PL 21.1 detect epitopes in the seminal fluids around the sperm. PL 15.2 also marks the cuticle of ejaculatory duct and the trachea. The outermost layer of core binds all of the IgG's tested.

The ejaculatory ducti is the organ where the final shape and structure of spermatophore is defined as secretions of reproductive accessory glands flow backwards. The wall of this duct is composed mainly of multilayered circular muscles surrounding a complex non-lamellate cuticle. Various surface structures (spines, cones, microtrichia-like structures, ribs) are asymmetrically distributed among the length of the duct. These complicated structures make their imprint in the secretory mass as it flows through the duct while the spermatophore is forming.

VITELLOGENIN OF THE HONEY BEE, *Apis mellifera*

D. Wheeler,[1] and J. Kawooya[2]

Department of Entomology,[1] and Department of Biochemistry,[2] University of Arizona, Tucson, AZ 85721

Vitellogenins in the order Hymenoptera appear to be fundamentally different from those in other insects in that they may consist of only a single, large apoprotein. Despite this unusual feature and the abundance of members of the Hymenoptera, the vitellogenins and vitellins of this order have been poorly characterized relative to other insect groups. We developed a protocol for isolating vitellogenin from the hemolymph of virgin honey bee queens. The three purification steps are density-gradient ultracentrifugation, ion-exchange chromatography and affinity chromatography. Honey bee vitellogenin is a VHDL with a density of about 1.28 g/ml. SDS-PAGE indicates that the apoprotein has a molecular weight of about 180,000. A variety of gel filtration columns, containing agarose and acrylamide-based media run under denaturing and non-denaturing conditions, point to a higher molecular weight of about 290,000. We conclude that apoprotein is not found as a dimer in its native state. The protein carries about 2% carbohydrate consisting almost entirely of mannose and about 7.5% lipid. Analysis of total amino acid composition showed high ASP and GLU content typical of other insect vitellogenins. The N-terminal amino acid sequence was determined, the first for any insect vitellogenin.

PURIFICATION AND INITIAL CHARACTERIZATION OF THE MAJOR CYTOCHROME P-450 FROM A PYRETHROID RESISTANT HOUSE FLY

G.D. Wheelock, and J.G. Scott

Institute for Comparative and Environmental Toxicology and Department of Entomology, Cornell University, Ithaca, NY

The LPR strain of house fly exhibits high levels of cytochrome P450 monooxygenase dependent pyrethroid resistance and constituitively expresses high levels of cytochrome P450s. We have purified the major cytochrome P450 from LPR flies to chromatographic homogeneity. Our procedure uses two HPLC steps to produce ~450 μg of purified cytochrome P450 in just two days. This preparation has a specific content of 14.4 nmol cytochrome P450/mg protein, a molecular weight of 54,300 by electrophoresis, a carboxy ferrocytochrome absorbance maximum at 447 nm and is termed cytochrome P450 lpr.

High titer polyclonal antiserum was developed against cytochrome P450 lpr (anti-cytochrome P450 lpr). This antiserum forms a single sharp precipitin line with both purified cytochrome P450 lpr and crude cytochrome P450 extract (i.e. microsomes) using rocket immunoelectrophoresis. Additionally, electrophoresis of purified cytochrome P450 lpr or LPR microsomes, followed by electroblotting and probing with anti-cytochrome P450 lpr (immunoblotting) reveals single immunologically reactive bands at the position of cytochrome P450 lpr in both the purified cytochrome P450 lpr and LPR microsome samples.

LPR microsome contain an average 0.72 nmol cytochrome P450 /mg protein. Cytochrome P450 lpr is 68% of total cytochrome P450 in LPR flies, determined by rocket immunoelectrophoresis and immunoblotting/ELISA. Microsomes (25 μg) from LPR and two insecticide susceptible house fly strains (S+, *aabys*) (nmol cytochrome P450/mg protein: S+=0.14, *aabys*=0.18) were assayed for cytochrome P450 lpr by immunoblotting. Cytochrome P450 lpr was barely detectable in the susceptible strains while LPR gave a strong immunoreaction. Elevated levels of total cytochrome P450 seen in LPR flies compared to susceptible flies thus appear due primarily to the overexpression of cytochrome P450 in LPR flies.

Cytochrome P450 lpr was also detected in several other insecticide resistant strains; additional evidence that LPR is an excellent model to elucidate the molecular and genetic basis of insect resistance.

A MALATHION-DEGRADING ENZYME IN THE MOSQUITO *Culex tarsalis*

S. Whyard, C. Tittiger, A.E.R. Downe, and V.K. Walker

Department of Biology, Queen's University, Kingston, Onatario, Canada, K7L 3N6

It is important to understand the biochemical basis for insecticide resistance since the control of pest insect populations has become increasingly difficult. In our malathion-resistant strain of *Culex tarsalis*, resistance appears to be due to an increased activity of a malathion carboxylesterase (MCE), which hydrolyses organophosphate malathion to less toxic, more easily excreted carboxylic acids. The resistant mosquitoes are 450 times more resistant to malathion and have 55 time more MCE activity than the susceptible strain. Crosses between the two populations showed that resistance was incompletely dominant; MCE activity and sensitivity to malathion in progeny were intermediate between the two parental strains. Resistance was stable without selection for 6 generations. The carboxylesterase inhibitor, triphyenylphosphate, acts as a strong synergist and increases the sensitivity to malathion by more than 100 times, suggesting that MCE plays a prominent role in the malathion resistance in our mosquitoes. MCE was localized predominantly to the midgut in larvae, and enzyme activity was present at all developmental stages. MCE has been purified from resistant insects and appears distinct from general esterases by its absence in gels stained for general esterases using naphthyl acetate as a substrate and by its specificity for malathion. To determine the molecular basis of malathion resistance in these mosquitoes, a genomic library from resistant adult *C. tarsalis* has been prepared. (Supported by the Natural Sciences and Engineering Research Council, Canada.)

cDNA AND GENE SEQUENCE OF *Manduca sexta* ARYLPHORIN: HOMOLOGY TO ARTHROPOD HEMOCYANINS

E. Willott, X.-Y. Wang, and M.A. Wells

Department of Biochemsitry, University of Arizona, Tucson, AZ 85721

The serum (storage) proteins produced by insect larvae at the end of the feeding cycle are hexameric blood proteins with one or more type of subunits. The cDNA and gene structure of the aromatic amino acid-rich larval serum protein arylphorin from the tobacco hornworm, *Manduca sexta*, has been determined. In *M. sexta* arylphorin there are two subunits α and β which have 686 and 687 amino acids, respectively, and whose amino acid sequences are 68% identical. The two genes, separated by 7.1 kb of chromosomal DNA, are transcribed in the same direction. Based on the alignment of the amino acid sequence, the rate of nucleotide substitution between the two coding regions predicts that the two genes diverged about 100 million years ago. Both genes contain 5 exons and the upstream region contains a sequence, TGATAAA, which is similar to a sequence found in other storage protein genes for which information is available. When the National Biomedical Research Foundation protein sequence data base was searched, it was found that the arylphorin showed significant similarity to the arthropod hemocyanisn, which are hexameric oxygen-carrying proteins. Based on the alignment of the sequence of *M. sexta* arylphorin and the hemocyanin from the spiny lobster (*Panulirus interruptus*), for which a 3.2 Å structure has been determined, it was observed that the highest concentration of conserved residues were found in those regions of the sequence which are involved in subunit interactions in the hexameric protein. It is

suggested that the insect storage proteins and the arthropod hemocyanins have evolved from a common ancestor. (Supported by NIH grant HL 39116.)

THE Δ-11 DESATURASE FROM THE PHEROMONE GLAND OF THE CABBAGE LOOPER MOTH

W.A. Wolf, and W.L. Roelofs

New York State Agricultural Experiment Station, Geneva, NY 14456

The first step in biosynthesis of cabbage looper moth *Trichoplusia ni* sex pheromone components from palmitic and stearic acids involves the introduction of a (Z) double bond in the 11-12 position. The desaturase that catalyzes this reaction has been partially purified by centrifugation and some of its properties determined. The enzyme is microsomal and requires NADH as a cofactor, unlike the more common Δ-9 desaturase which uses NADPH. We will report on the further purification and characterization of this membrane-bound protein. The purified protein will be compared with proteins produced by *in vitro* transcription of mRNA obtained from cabbage looper sex pheromone gland.

THE STRUCTURE OF *Acheta domesticus* AKH AND THE RESPONSE TO THE HORMONE

J. Woodring,[1] and G. Gäde[2]

[1] Department of Zoology-Physiology, LSU, Baton Rouge, LA 70803
[2] Department of Zoology, University of Cape Town, Private Bag Rondebosch 7700, Republic of South Africa

The neuropeptide derived from the corpora cardiaca of *Acheta domesticus* showed strong adipokinetic activity when injected into *A. domesticus*. The structure of the *Acheta* peptide is identical to the peptide Grb-AKH described previously from *Gryllus bimaculatus*, which is: pGlu-Val-Asn-Phe-Ser-Thr-Gly-Trp-NH_2. Synthetic Grb-AKH activates glycogen phosphorylase in the fat body of *A. domesticus*. Dose response and time response curves for plasma lipids upon injection of Grb-AKH are presented and compared to the responses obtained with Lom-AKH-I (Locusta). The response of the fat body to Grb-AKH increased during the first 4 days after ecdysis in both the last larval instar and the adult stage.

Research carried out at the Henirich-Heine-Universität, Zoologie IV (Tierphysiologie), 4000 Düsseldorf, Federal Republic of Germany. J.W. supported by Alexander von Humboldt Fellowship, G.G. supported by a grant and a Heisenberg Fellowship from the Deutsche Forschungsgemeinschaft.

PHYSIOLOGICAL CONTRASTS BETWEEN PREVITELLOGENIC AND VITELLOGENIC FOLLICLES

R.I. Woodruff,[1] and W.H. Telfer[2]

[1] Department of Biology, West Chester University, West Chester, PA 19383
[2] Department of Biology, University of Pennsylvania, Philadelphia, PA 19194-6018

Previtellogenic development of ovarian follicles in insects leads to a relatively stable and quiescent state that is terminated by hormonal activation in many species, and apparently spontaneously in others. In one of the latter, *Hyalophora cecropia*, we have compared

previtellogenic and early vitellogenic follicles and found that activation involves a number of additional physiological changes. 1) Oocyte and nurse cell membranes hyperpolarize, adding an azide and vanadate-sensitive component to a basal potential that is unaffected by either of these inhibitors. 2) Hyperpolarization is accompanied by a rise in cytoplasmic pH, due to an azide-sensitive proton extrusion mechanism, and 3) is also closely associated with the onset of electrical coupling between cells within follicles, as well as between adjacent follicles. 4) Since hyperpolarization is stronger in the nurse cells, it gives rise to an electrical potential across the cytoplasmic bridges that connect these cells. The transbridge potential is reversibly abolished by both azide and vanadate. 5) The follicle nearly trebles its volume in a several hour period, apparently due to osmotic swelling. 6) Uridine incorporation shuts down in the germinal vesicle, and accelerates in the nuclei of the nurse cells and the follicular epithelium. And finally, 7) endocytotic yolk formation begins. Plausible speculations are possible on a causal relationship linking most of these changes to ion pump activity in the oocyte and nurse cell membranes. (Supported by NSF grant DB-18617.)

SPECIFICITY AND ROLE OF ALKALINE PHOSPHATASE ISOZYMES IN THE MIDGUT OF THE SILKWORM, *Bombyx mori*

H. Yamamoto, S. Takeda, M. Azuma, and M. Eguchi

Department of Applied Biology, Kyoto Institute of Technology,
Matsugasaki, Kyoto 606, Japan

Alkaline phosphatase isozymes, membrane-bound (m-ALP) and soluble (s-ALP) forms occur in the *Bombyx* midgut. Using normal and mutant strains deficient in each isozyme, we studied the property of purified isozymes and compared with mammalian ALPs. Both forms were found to be monomer, and the molecular weights were estimated to be 58 kDa for m-ALP and 61 K for s-ALP by SDS-polyacrylamide gel electrophoresis. The m-ALP was extremely stable even in pH 10-12 which corresponds to the pH value of *Bombyx* midgut lumen. The activity of both isozymes was inhibited by L-cysteine strongly but not by other amino acids. Above properties are clearly different from those of mammalian ALPs (Okada et al., J. Comp. Phsyiol., 1989). The Zn^{2+} was most effective for restoration of activity after EDTA treatment. Moveover, both isozymes were immunologically different. Immunohistochemistry revealed that the m-ALP was localized at the brush border of columnar cells, whereas the s-Alp was localized at the apical surface of goblet cells (Azuma and Eguchi, J. Exp. Zool., 1989). We detected ATPase activity in the purified s-ALP preparation; Mg^{2+} was essential from the ATPase activity and the activity also increase with $KHCO_3$ but not with KCl. By surveying 60 inbred *Bombyx* strains, electrophoretic variants of m-ALP were found and these variants were immunologically identical. To elucidate the structure and expression of ALP genes, DNA cloning of both isozymes is in progress in our laboratory.

A NEW MEMBER OF A LOW MOLECULAR WEIGHT HEMOLYMPH PROTEIN FAMILY FROM *Locusta migratoria*

J.-Z. Zhang, and G.R. Wyatt

Department of Biology, Queen's University, Kingston, Onatario, K7L 3N6, Canada

From hemolymph of *Locusta migratoria* we have purified a low molecular weight (21 kDa) protein that is produced in the fat body and taken up into oocytes during vitellogenesis. The 21K protein comigrates on SDS PAGE with locust apolipophorin III (Zhang and Wyatt, 1988, J. Biol. Chem. 263:10568,) and the 19K protein of Kanost et al., (1988, Arch. Insect Biochem. Physiol. 8:203), but is easily separable by native PAGE or ion exchange. Its N-terminal sequence has 17 out of 26 amino acids identical with those of the 19K protein but its contents of arginine and lysine are much higher. The native form is monomeric and contains some carbohydrate. It shows no immunological cross reaction with apoLpIII, 19K protein, or vitellogenin. It is produced only in female adult locusts. After treatment with precocene to eliminate JH production, 21K protein does not appear, but synthesis can then be induced by administration of a JH mimic such as methoprene, which indicates that synthesis is coordinated in time with induction of vitellogenesis, but the level is much lower. Despite its smaller size and different amino acid composition, these characteristics suggest some physiological similarity to the microvitellogenins of Lepidoptera. (Supported by grants for NSERC of Canada and U.S. NIH).

ADIPOKINETIC HORMONE AND GLYCOGEN PHOSPHORYLASE ACTIVATING HORMONE OF *Manduca sexta* ARE IDENTICAL

R. Ziegler, K. Eckart, and J.H. Law

Department of Biochemistry, University of Arizona, Tucson, AZ
Max-Planck-Institut, Exp. Medizin, Goettingen

In *Manduca sexta* an adipokinetic hormone from the corpora cardiaca mobilizes lipids from the fat body during flight. Injection of adipokinetic hormone induces a strong increase in the concentration of hemolymph lipids and the conversion of a high density lipoprotein to a low density lipoprotein. This adipokinetic hormone is a nonapeptide of the sequence PGlu-Leu-Thr-Phe-Thr-Ser-Ser-Trp-GlyNH_2 (Ziegler, et al., 1985, Biochem. Biophys. Res. Commun. 133:337-342). In larvae there is a peptide hormone in the corpora cardiaca which activates fat body glycogen phosphorylase during starvation. We isolated the larval hormone by reverse phase HPLC and sequenced it by fast atom bombardment tandem mass spectrometry. The larval peptide is identical with adipokinetic hormone of the adults. Thus, the adipokinetic hormone of *M. sexta* has different functions within adults and in larvae. In adults it mobilizes lipids during flight, while in larvae it mobilizes carbohydrates during starvation. (Supported by DFG Zi 135/9-1 and HIH A126905 and GM29238)

HOMEOTIC GENES OF THE RED FLOUR BEETLE

R.W. Beeman, S.J. Brown, J.J. Stuart, and R.E. Denell

U.S. Grain Market Research Laboratory, and Kansas State University, Manhattan, KS

We recently began studies of the segmentation and homeotic genes of *Tribolium castaneum* to allow comparisons with those of Drosophila. Important homeotic genes are clustered in a single complex (the HOM-C), in which we have isolated over 80 new mutations. The adult homeotic phenotypes of these variants differ from those of Drosophila in many interesting and unpredicted ways.[1] For example, mutants of the *Abdominal* (*A*) gene show bidirectional transformations of anterior and posterior abdominal segments to resemble those in the middle. Studies of the embryonic and larval lethal phenotypes are underway, aided by the rich array of segment-specific features expressed by beetle larvae. *A* homozygotes dies as late embryos showing only anterior abdominal transformations.

T. castaneum has a genome size (.21 pg) and organization similar to Drosophila. We have used a Drosophila *Antennapedia* homeo box probe to clone complementary fragments from a beetle genomic library. Thus far sequencing has identified fragments homologous to the Drosophila *abdominal-A* and *Ultrabithorax* genes, and an RFLP shows that *abd-A* and *A* are homologous.

Antibodies against the Drosophila Antennapedia and engrailed proteins recognize conserved nuclear antigens in beetle embryos, and *prothoraxless* embryos are negative with respect to the former. The engrailed immunostaining pattern in the epidermis and CNS is very similar to Drosophila, but in this intermediate germ band insect engrailed stripes appear sequentially during abdominal segmentation.

Beeman, R.W., et. al. Developmental Biology 133:196

CYTOCHROME P450 IN PLANT-INSECT INTERACTIONS: INDUCTIONS AND DEDUCTIONS

M.R. Berenbaum

Department of Entomology, 320 Morrill Hall, University of Illinois, 505 S. Goodwin, Urbana, IL 61801-3795

Although microsomal cytochrome P450-monooxygenases are known to play a role in the evolution of resistance to synthetic organic insecticides, their importance in adaptation to hostplant allelochemistry by phytophagous insects is largely unexplored. In oligophagous species associated with umbelliferous hostplants, particular P450s with narrow substrate specificity metabolize hostplant furanocoumarins. In the oligophage *Papilio polyxenes*, P450-mediated metabolism of the furanocoumarin xanthotoxin is inducible and involves only a subset of P450 isozymes; xanthotoxin isomers and other furanocoumarins are metabolized with significantly lower efficiency. P450 induction by xanthotoxin is associated with the appearance of a 51.2 kD polypeptide that crossreacts with mammalian anti-P450 antibody and that is not induced by other known P450 inducers. Molecular genetic techniques are being used to characterize and clone the gene(s) responsible for furanocoumarin metabolism; the furanocoumarin-induced P450 clone

and the monoclonal antibodies to this P450 can be used to determine if the P450s in other umbellifer specialists are homologous or are products of parallel evolution of different ancestral P450s. Within the Papilionidae, for example, umbellifer-feeding is thought to have arisen independently three times; comparing furanocoumarin-metabolizing P450s and the genes regulating them can provide insight into the evolutionary mechanisms underlying host shifts and specialization.

A LARVAL SPECIFIC LIPOPROTEIN IN *Musca domestica*

A.G. De Bianchi, and M. de L. Capurro

Department of Biochemistry, University of São Paulo, São Paulo 01498, CP 20780 Brazil

During the purification of larval *M. domestica* lipophorin a contaminant lipoprotein floats together with it in a potassium bromide density gradient. This protein is separated from lipophorin in a glycerol gradient ultracentrifugation. This lipoprotein must have a higher molecular weight than lipophorin since it goes to the bottom of the glycerol gradient centrifugation tubes ahead of lipophorin. This lipoprotein does not occur in adults thus it was denominated larval lipoprotein (LLP). The LLP has a density equal to 1.134 ± 0.007g/ml and is composed of at least five types of apolipoproteins (ApoLLP I to ApoLLP V) with molecular weights equal to 48,000 ± 2,500, 41,000 ± 2,100, 26,900 ± 600, 24,500 ± 600 and 21,899 ± 600.

Preliminary data suggests that LLP lipidic moiety is composed mainly of diacylglycerols (1,2 and 1,3) and cholesterol. Phospholipids and hydrocarbons are also present but in minor relative amounts. Fat bodies from feeding larvae incubated *in vitro* with [^{35}S]-methionine synthesizes LLP that is secreted to the incubation medium. The newly synthesized LLP has the same density as mature LLP. This lipoprotein resembles some mammalian lipoproteins due to the fact that it is composed by several low molecular weight apolipoproteins. However, the lipidic moiety is completely different in mammalian and larval insect lipoprotein.

HEREDITARY VIRUSES OF *Heliothis*?: CHROMATIN ASSOCIATED VIRUS-LIKE PARTICLES IN TESTES OF THREE SPECIES OF *Heliothis*, *Heliocoverpa asulta*, AND INTERSPECIFIC HYBRIDS OF THE ABOVE

M.E. DeGrugillier

Biosciences Research Laboratory, USDA, ARS, Fargo, ND 58105

Virus-like particles, (VLP's) were found associated with the chromatin of spermatocyst cell nuclei in adult *Heliothis virescens*, *H. subflexa*, *H. zea*, and *Heliocoverpa asulta*. In addition, the 50-55nm spherical particles were present in spermatocyst and follicle cell nuclei of testes of F_1 males derived from interspecific crosses involving *H. virescens* x *H. subflexa* and *H. asulta* x *H. zea*. The VLP's were also found in testes of 150th generation backcross (BC) males that were derived from the hybridization of *H. virescens* x *H. subflexa*. The chromatin associated VLP'S suggests the presence of a hereditary virus and this virus may prove to be the basis for the sterility observed in BC males.

ANALYSIS OF A MUTATION IN *Bombyx mori* THAT AFFECTS CHORION ULTRASTRUCTURE

D. Gautreau, M.G. Alexopoulou, and M.R. Goldsmith

Department of Zoology, University of Rhode Island, Kingston, RI 02881-0816

Gr^{16} is a spontaneous dominant mutation of the domestic silkmoth, *Bombyx mori*, in which the helicoidal array of fibers that comprises the bulk inner chorion is perpendicular rather than parallel to the egg surface. The mutation maps between the two known chorion structural gene clusters. The first signs of abnormality appear during early chorion formation (G.D. Mazur, personal communication), suggesting that the mutation affects a critical step in the assembly of the initial extracellular framework. To study the molecular basis of the mutation, we compared mature chorion protein composition, kinetics of protein synthesis, and the gross structure of the early chorion locus in the homozygous mutant, its corresponding wild type, and the heterozygote. Two-dimensional protein patterns of mature chorions were identical, indicating that *Gr*^{16} expresses all of the major chorion components at normal levels. However, two pairs of minor, early proteins showed reciprocal differences in relative labeling intensities: one pair labeled more intensely in the wild type, while the other was more prominent in the homozygous mutant. These two pairs of proteins had similar molecular weights but different isoelectric points, suggesting that they are related. Preliminary pulse-chase labeling patterns were consistent with the hypothesis that the mutation affects post-translational modification of the proteins in question. Restriction analyses of genomic DNA using early chorion genes as probes revealed no difference between mutant and wild type, indicating that the mutant lacks major chromosome defects. We will present models for the molecular level at which the mutation may act, in light of these observations.

PROPERTIES AND CLONING OF LOW ABUNDANCE ENZYME OF HIGH SIGNIFICANCE: THE JUVENILE HORMONE ESTERASE FROM *Heliothis virescenes*

B. Hammock,[1,2] T. Hanzlik,[1] L. Harshman,[1] V. Wroblewski,[1] M. Philpott,[1] A. Szekacs,[1] B. Bonning,[2] R. Possee2

[1] Departments of Entomology and Environmental Toxicology, University of California, Davis, CA 95616
[2] NERC Institute of Virology and Environmental Microbiology, Mansfield Road, Oxford, OX1 3SR, UK

In lepidopterous larvae the reduction in juvenile hormone titer which in turn leads to a decrease in feeding and pupation is caused by a concerted reduction in biosyntheses and increase degradation by a highly specific enzyme known as juvenile hormone esterase (JHE). This enzyme has been purified from several species by affinity chromatography and its 3 Kbp message cloned from *H. virescenes*. Using a monospecific antibody and oligonucleotide probe for JHE, it was found that the presence of protein and message in insect tissues correlates with the presence of JHE activity. The gene for JHE has been placed into several baculovirus vectors and these systems have been found to produce high levels of JHE *in vitro* and moderate levels *in vivo*. The ease of assay of this enzyme and its high kCAT/KM ration indicate that this will be a useful reporter enzyme for studying expression systems. The expressed enzyme has similar biochemical, immunochemical and catalytic properties to the natural enzyme. When injected, both the natural and the expressed esterases have been shown to have anti-juvenile hormone effects. Thus, the JHE is a desirable target gene for the control of insects in addition to being of fundamental interest in developmental biology.

CLONING AND CHARACTERIZATION OF MOSQUITO ACETYLCHOLINESTERASE GENES

C.A. Malcom and L.M.C. Hall

School of Biological Sciences, Queen Mary College, University of London, Mile End Road, London E1 4NS and Department of Medical Microbiology, London Hospital Medical College, University of London, Turner St., London E1 2AD

Acetylcholinesterase (AChE) is the target site for organophosphate and carbamate insecticides. In a number of insect pests including mosquitoes altered forms of the enzyme have been found which confer broad spectrum insecticide resistance. Within this context the results of studies on mosquito AChE molecular biology are presented. The AChE gene was cloned from the major malaria vector *Anopheles stephensi*, by homology to a *Drosophila melanogaster* AChE cDNA clone (Hall and Spierer, 1986).

Sequencing of the mosquito AChE gene indicated 70% homology with the *D. melanogaster* gene at the amino acid level, with complete conservation of the active site. The intron exon boundaries corresponded exactly, but the genome organization was found to be much more compact, with each of the introns being 100 bps or less.

In situ hybridization to ovarian nurse cell polytene chromosomes identified two locations. One was close to the telomere of chromosome 2R, a similar position to that previously established for the AChE gene in *An. albimanus* (Kaiser and Seawright, 1978) the other was close to the centromere of chromosome 3L.

Hall, L.M.C. and Spierer, P. (1986) EMBO J. 5:2949-2954.

Kaiser, P.E., Seawright, J.A. and Joselyn, D.J. (1979) Canadian Journal of Genetic Cytology, 21:201-211.

TRANSFORMATION OF THE MOSQUITO *Aedes aegypti* USING THE "P" ELEMENT, ISOLATED FROM *Drosophila melanogaster*

A. Morris, P. Eggleston, and J. Crampton

Wolfson Unit of Molecular Genetics, Liverpool School of Tropical Medicine, Liverpool L3 5QA, U.K.

The "P" element, a transposable genetic element isolated from *Drosophila melanogaster*, is being used as a DNA transformation vector in the manipulation of the genome of the mosquito *Aedes aegypti*. A micro-injection system has been developed for the introduction of DNA into the developing embryo. Genomic DNA analysis of surviving adults and their progeny has revealed the integration of injected DNA into the chromosomes of these individuals. There is growing evidence that these events are not mediated by transposition, although the role of the P element remains equivocal. Further characterization of similar transformants should determine the precise nature of these integration events. The expression of the P element transposase gene in *A. aegypti* is also being studied. This gene, found on intact P elements, is co-injected with the vector plasmid and its protein product is essential in the mechanism of transposition. An analysis of the processing of this gene in the mosquito embryo may lead to the subsequent use of modified P element constructs which are able to transpose in non-drosophilids.

A BACULOVIRUS BLOCKS MOLTING OF ITS INSECT HOST BY EXPRESSING AN ECDYSTEROID UDP-GLUCOSYL TRANSFERASE GENE

D.R. O'Reilly and L.K. Miller

Departments of Entomology and Genetics, University of Georgia, Athens, GA 30602

A newly identified gene (*egt*) of the baculovirus *Autographa californica* nuclear polyhedrosis virus (AcMNPV) has been characterized. Two 5'-coterminal mRNAs are transcribed from this gene early after infection of insect cells in tissue culture. The predicted amino acid sequence of *egt* reflects homology to several UDP-glucuronosyl transferases and at least one UDP-glucosyl transferase. Genetic and biochemical studies confirm that the *egt* gene encodes a UDP-glucosyl transferase which catalyses the transfer of glucose from UDP-glucose to various ecdysteroids. The enzyme is secreted and accumulates in the extracellular medium of infected cells in tissue culture. Expression of an ecdysteroid UDP-glucosyl transferase allows the virus to interfere with normal insect development so that molting is blocked in the infected larvae.

SYNTHESIS AND PROCESSING OF NEUROPEPTIDE PRECURSORS: MOLECULAR AND BIOCHEMICAL APPROACHES

M. O'Shea, S. Hekimi, and M.-F. Schulz

Laboratory of Cell Biology, University of London, New College, Egham, Surrey, UK

Two adipokinetic hormone (AKH I and AKH II) are found in the glandular lobe of the *Corpus Cardiacum* of the locust. We have studied the biosynthesis of these hormones by a combination of cDNA cloning, *in vitro* translation and protein chemistry. Two mRNAs, (A and B) have been cloned and sequenced. These give rise to two proteins, which we call pre-A-chain and pre-B-chain respectively. After removal of a signal peptide from each, these proteins associate with one another to form three dimer precursors (P1, P2 and P3). P1 is a homodimer of two A-chains. Each A-chain consists of AKH I, a processing site and a 28 residue peptide called the α-chain. The B-chain consists of AKH II, a processing site and a similar 28 residue peptide call the β-chain. Processing of the two homo- and one heterodimer of these peptides results in the generation of the AKH I and AKH II peptides and the three AKH Precursor Related Peptides (APRPs). The APRPs are the homo- and heterodimers formed from the α and β chain subunits. The regulatory implications of dimerisation of peptides at the level of precursors will be discussed, as will the control during postembryonic development of peptide expression and precursor biosynthesis.

DEVELOPMENT AND RELEASE OF GENETICALLY ENGINEERED VIRAL INSECTICIDES: A PROGRESS REPORT 1986-1989

R.D. Possee and D.L. Bishop

NERC Institute of Virology and Environmental Microbiology, Mansfield Road, Oxford OX1 3SR, UK

Baculoviruses have been used for many years as cost-effective, biological insecticides. They have the advantages of only infecting arthropods and a degree of species specificity which permits targeting of particular pest insects. They resist inactivation in the environment because

of a crystalline, proteinaceous matrix surrounding the virus particles. However, a baculovirus infection may take several days to kill the host, thus posing a serious disadvantage in comparison to faster acting chemical insecticides. Genetic engineering of baculoviruses presents the opportunity to improve the natural virus insecticides by expression insecticidal gene products such as toxins or hormones which potentially can hasten the death of the host. However, such endeavors raise serious issues concerning the safety of genetically modified products after their release into the environment.

Therefore, in a cautious and progressive program of research the *Autographa californica* nuclear polyhedrosis virus (AcNPV) has been genetically modified to facilitate laboratory and field risk assessment analyses to evaluate the consequences of the release of genetically engineered viruses into the environment. Factors such as the alteration of host-range, persistence in soil and susceptibility to ultra-violet radiation have been studied. The program began in 1986 with the release of genetically marked AcNPV to permit the persistence of a virus within the environment to be assessed. In 1987 a virus lacking the protective polyhedron structure was released to confirm that a virus insecticide could be designed to "self-destruct" after release into the environment and thus reduce the likelihood of unwanted spread. In more recent studies, self destruct viruses expressing beta-galactosidase, *Bacillus thuringiensis* delta-endotoxin and insect juvenile hormone esterase have been analysed. Data will be presented describing the field studies and the current status of the program to produce a new generation of virus insecticides.

ISOLATION, CHARACTERIZATION AND CLONING OF CYCLODIENE INSECTICIDE RESISTANCE IN Drosophila melanogaster

R.T. Roush and R.H. ffrench-Constant

Department of Entomology, Comstock Hall, Cornell University, Ithaca, NY 14853

We have isolated a single gene conferring high levels of resistance to cyclodiene insecticides from field-collected populations of *Drosophila melanogaster*. Apparently, cyclodienes used against pests in crops such as tomatoes and peaches have selected for this gene. The resistance gene has been localized by recombination and deficiency mapping to a single letter band on the left arm of chromosome 3. Striking similarities in chromosomal location and spectrum of resistance to various cyclodiene insecticides suggest that this gene is homologous to cyclodiene resistance in other insects. Progress in cloning of the gene via chromosomal walking and P-element mediated mutagenesis will be described. The possible mechanisms for the resistance, including that resistance is conferred by an insensitive GABA receptor, will be discussed. The potential uses of this and other *Drosophila* genes for studies of pesticide resistance in other species are extensive.

ISOLATION AND IDENTIFICATION OF NEUROHORMONES FROM *Manduca sexta*

D.A. Schooley,[1,2] R.L. Carney,[1] H.Kataoka,[3] S.J. Kramer,[1] J.P. Li,[1] A. Toschi,[1] R. Troetschler[1]

[1] Sandoz Crop Protection, Palo Alto, CA 94304
[2] Current address: University of Nevada, Reno, NV 89557
[3] University of Tokyo, Tokyo 113, Japan.

We recently developed a scheme for isolating the eclosion hormone of *Manduca sexta* from "trimmed heads" of pharate adult animals. Subsequently, we isolated an adult-specific allatotropin from side fractions. Initial attempts to isolate a diuretic hormone from other side

fractions were encouraging, but the activity was lost during isolation. After modifications to the liquid chromatograph and the isolation procedure, we successfully isolated DH from an extract of another 10,000 trimmed heads. We will review the sequences of these three *M. sexta* neurohormones and what is currently known of the structure/activity relationships for the allatotropin. We will also discuss the strategy of the isolation scheme, which may be rather general for basic and relatively acidic neuropeptides.

DEVELOPMENT REGULATION OF SILK GENE EXPRESSION IN *Bombyx mori*

Y. Suzuki, S. Takiya, T. Suzuki, C.-C. Hui, and K. Matsuno

National Institute for Basic Biology, Okazaki, 444 Japan

The fibroin gene and the sericin-1 gene are expressed specifically in the posterior silk gland and the middle silk gland, respectively. The transcript of the fibroin gene was first detected in the embryos at around stage 25 when the organogenesis of the silk gland is completed, and was repeatedly detected in the feeding stages but not in the molting stages of larval development. The sericin-1 gene was found to be expressed at the later stages of larval development.

Cell-free transcription systems derived from various tissues of different developmental stages and various cultured cells have been developed and used to identify the transcriptional control elements of the fibroin and sericin-1 genes. DNase I footprinting and gel shift assays were also used to characterize the *trans*-acting factors of the *cis*-acting elements. Based on these results, the cloning of cDNA candidates for these *trans*-acting factors has been carried out.

Information currently available for these gene systems will be summarized and possible models of the tissue-specific regulation of silk gene expression will be discussed.

ENGINEERING OF BACULOVIRUS VECTORS FOR THE EXPRESSION OF FOREIGN GENES IN INSECTS

J.M. Vlak, A. Schouten, P.W. Roelvink, J.W.M. Martens, and D. Zuidema

Department of Virology, Agricultural University, Binnenhaven 11, 6709 PD Wageningen, the Netherlands

Baculovirus are attractive biological control agents of insect pests in agriculture and forestry. They are also exploited as biotechnological tools for high-level expression of foreign genes in a eukaryotic environment. Baculoviruses contain two genes, that are expressed abundantly late after infection: polyhedrin, coding for the matrix protein of polyhedra, and p10, the function of which is unknown so far. Polyhedra protect the virus from decay in the abiotic environment and are essential for the initiation of infection in insects. The p10 protein is not required for virus replication, polyhedron formation or insecticidal activity. Introduction of foreign genes into baculoviruses under the control of the p10 promoter is therefore attractive for production of recombinant proteins in insects. The isolation of p10 recombinants however is difficult, since there is no easy screening or selection system, in contrast to polyhedrin recombinants which can be detected visually by their polyhedrin-negative appearance. We have now developed a novel and convenient vector system for the selection of p10 recombinants for the expression of foreign genes. A special transfer vector was designed containing the flanking regions of the p10 gene to enhance recombination, a unique cloning site for the insertion of the gene of choice, and a special reporter gene cassette. The cassette contains a LacZ gene under the control of a heat-shock promoter. Recombinants containing a foreign gene can now be conveniently selected by the expression of the reporter gene (beta-galactosidase). This screening system is also less dependent of a high frequency of recombination, due to the sensitive detection system. A major drawback to a wide-spread use of baculoviruses as control agents is their slow speed-of-action.

Upon infection insects stop feeding only after a few days, where immediate action is required. Genetic manipulation of these viruses by introduction of genes for insect toxins, hormones or metabolic enzymes may increase the speed of action. This novel screening system has been used to select baculovirus recombinants expressing such genes.

REGULATING GENES FOR METAMORPHOSIS: CONCEPTS AND RESULTS

J.H. Willis

Department of Entomology, University of Illinois, Urbana, IL 61801

In 1940, V.B. Wigglesworth published a paper where "a new hypothesis is put forward to explain the action of the inhibitory hormone in controlling metamorphosis" and he concluded by renaming the inhibitory hormone, juvenile hormone. In the intervening half century his seminal observations have been confirmed in diverse species, his "enzyme systems" have been replaced by gene sets, and molecular biology has come of age. We are at last able to make and to test specific predictions on how the genes whose products underlie the morphological manifestations of metamorphosis are controlled. This paper will discuss experiments designed to make sense of the recent findings from several laboratories that many juvenoid analogues fail to compete with natural juvenile hormones in receptor assays. It will present a scheme for constructing cDNA libraries enriched in messages that code for proteins that intercede between the genes that are the primary targets for ecdysteroid action and the genes for cuticular proteins that show so much regional specificity in their activity. It will urge the implementation of experiments designed to permit us to distinguish between a major anti-metamorphic action of juvenoids and a minor role regulating the levels of activity of many genes. In this context, it will explain why the findings that a gene has a *cis*-acting element capable of binding a JH-receptor complex need not be relevant to elucidating the control of metamorphosis.

Wigglesworth, V.B. (1940) Journal of Experimental Biology, 17:201-222.

RECEPTOR-MEDIATED ENDOCYTOSIS OF INSECT YOLK PROTEINS

H.-J. Ferenz

Insect Physiology Group, Dept. Biology, University of Oldenburg,
P.O. Box 2503, D-2900 Oldenburg, Federal Republic of Germany

The major source of nutrients for the developing embryo of all oviparous insects are the egg yolk proteins (vitellins). Their precursors, the vitellogenins, are usually synthesized in the fatbody, secreted into the haemolymph and selectively taken up by developing oocytes. The molecular basis of the selective endocytosis of insect vitellogenin was recently studied in a few insect species including *Locusta migratoria*. In vitro-studies with intact oocytes and more straight-forward binding studies with isolated ovarian membranes demonstrated the presence of a receptor system with high affinity for vitellogenin facilitating the selective and specific endocytosis of the yolk protein. In all species studied the equilibrium dissociation constant k is in the range of 10 M. The vitellogenin receptor from oocytes of *L. migratoria* could be solubilized with the detergent octylglucoside and purified by affinity chromatography retaining its binding properties. The locust vitellogenin receptor was identified as a 156 kDa glycoprotein with a pI of 3.55. It binds to positively charged lysine residues of the vitellogenin molecule; chemically modified vitellogenin cannot be recognized. Immunocytological studies with antibodies against the vitellogenin receptor demonstrated its presence in the oocyte membrane. However, the mechanism of this receptor-mediated endocytosis including the specific binding of vitellogenin and the events after internalization of the receptor-ligand complex need further clarification.

ANALYSIS OF THE INTEGRATION OF THE GENOME OF A SEGMENTED DNA VIRUS IN THE HOST INSECT'S GENOME

J.-A.G.W. Fleming

Deptartment of Entomology and Institute for Biosciences and Technology, Texas A&M University, College Station, TX 77843

The polydnavirus *Campoletis sonorensis* virus (CsV) is a primary component of the constitutive microflora of the oviduct of the ichneumonid wasp *C. sonorensis* and appears to be essential to the reproductive success of the endoparasitic wasp since it compromises the immune response of lepidopteran host larvae. CsV appears to be vertically transmitted in wasps. We provide evidence that several, if not all, of the 28 supercoiled viral DNAs in the CsV genome are integrated in wasp chromosomal DNA, making CsV the first documented integrated DNA, nonretroviral virus of insects. In Southern blots of wasp chromosomal DNA probed with plasmids representing complete viral superhelices B, H, M, O^1 and W (6.6, 8.4, 10.8, 11.2 and 15.8 kbp, resp.), these viral DNAs are detected as offsize restriction fragments. Cloned wasp DNAs which hybridize with viral DNA B were isolated from an EMBL3-*C. sonorensis* genomic library and compared with extrachromosomal viral DNA B by Southern blot and nucleotide sequence analyses. Viral DNA B is covalently linked to wasp DNA and is integrated without significant rearrangement. Preliminary data suggests that additional viral DNAs also are integrated regardless of whether they are related to DNA B. Sequences found at the junction of B and wasp DNA were related to sequences near the termini of the DNA B member of gene family of 540 bp repeat elements. To determine whether other 540 bp repeat elements which occur on most or all CsV DNA segments are associated with junctions, the integration sites were mapped on several additional viral DNAs on which the repeat elements have been mapped previously. The nucleotide sequence of these regions is being determined and compared with the published sequences of repeat elements found in these additional viral DNAs.

AUTHOR INDEX

INDEX